รายงานวิจัยฉบับสมบูรณ์

โครงการ ระบบภูมิคุ้มกันอินเนตในช่องปากที่มีต่อไวรัส

โดย รังสินี มหานนท์ และคณะ

กรกฎาคม 2556

สัญญาเลขที่ BRG5380011

รายงานวิจัยฉบับสมบูรณ์

โครงการ ระบบภูมิคุ้มกันอินเนตในช่องปากที่มีต่อไวรัส

คณะผู้วิจัย	สังกัด
รศ.ทญ.ดร.รังสินี มหานนท์	ภาควิชาปริทันตวิทยา คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ผศ.พญ.อรุณี ธิติธัญญานนท์	ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล
ผศ.ทญ.ดร.เกศกัญญา สัพพะเลข	ภาควิชาศัลยศาสตร์ คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ผศ.ทญ.ดร.กนกพร พะลัง	ภาควิชาเวชศาสตร์ช่องปาก คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ผศ.ทญ.ดร.พรพรรณ พิบูลย์รัตนกิจ	ภาควิชาเวชศาสตร์ช่องปาก คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
อ.ทญ.ภัทรนฤน กาญจนบุษย์	ภาควิชาเวชศาสตร์ช่องปาก คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและจุฬาลงกรณ์มหาวิทยาลัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. และจุฬาลงกรณ์มหาวิทยาลัยไม่จำเป็นต้องเห็นด้วยเสมอไป)

Abstract

Although oral mucosal tissue is continually challenged by microbial plaque, it is generally maintained in a healthy state. To understand the basis for this, we investigated innate antiviral immunity in human oral mucosal tissue. Immunostaining data showed that MxA, a potent anti-viral protein, was clearly observed in the epithelial layer of healthy and diseased periodontal tissue. On the other hand, this protein was minimally expressed in the epithelial layer of normal buccal mucosa and oral lichen planus. In periodontal tissue, epithelial MxA immunoreactivity seemed to be stronger in basal and spinous layers than outermost layer of oral epithelium. The experiments were then focused using periodontal tissue model. Immunostaining data consistently showed higher MxA protein expression in the epithelial layer of healthy periodontal tissue as compared with tissue with periodontitis. Human MxA is thought to be induced by type I and III IFNs but neither cytokine type was detected in healthy periodontal tissues. Treatment *in vitro* of primary human gingival epithelial cells (HGECs) with α-defensins, but not with the antimicrobial peptides β-defensins or LL37, led to MxA protein expression. α-defensin was also detected in healthy periodontal tissue. In addition, MxA in α-defensin-treated HGECs was associated with protection against avian influenza H5N1 infection and silencing of the MxA gene using MxA-targeted-siRNA abolished this antiviral activity. To our knowledge, this is the first study to uncover a novel pathway of human MxA induction, which is initiated by an endogenous antimicrobial peptide, namely α-defensin. This pathway may play an important role in the first line of antiviral defense in periodontal tissue.

Keywords: α-defensin, MxA, Homeostasis, Periodontal tissue, antivirus

บทคัดย่อ

แม้ว่าเนื้อเยื่อบุช่องปากจะถูกกระตุ้นอย่างต่อเนื่องโดยจุลชีพในคราบจุลินทรีย์ แต่เนื้อเยื่อบุช่องปากยังคงรักษาสภาพปกติไว้ได้โดยทั่วไป เพื่อที่จะเข้าใจถึงกระบวนการดังกล่าว คณะผู้วิจัยจึงทำการศึกษาระบบภูมิคุ้มกันโดยกำเนิดต่อไวรัสในเนื้อเยื่อบุช่องปากมนุษย์ ข้อมูลการย้อมทางด้านอิมมูโนวิทยาแสดงให้เห็นว่า MxA ซึ่งเป็นโปรตีนที่สามารถต่อต้านไวรัสถูกพบอย่างชัดเจนในชั้นของเยื่อบุผิวของชิ้นเนื้อเยื่อปริทันต์ที่ปกติและที่เป็นโรค ในทางตรงข้ามพบการแสดงออกของโปรตีนชนิดนี้ระดับต่ำในชั้นของเยื่อบุผิวของชิ้นเนื้อเยื่อบุกระพุ้งแก้มที่ปกติและชิ้นเนื้อของรอยโรคไลเคนพลานัส ในเนื่อเยื่อปริทันต์ การแสดงออกของ MxA ในเยื่อบุผิวดูเหมือนว่าจะชัดเจนในชั้นเบเซิลและชั้นสไปนัสมากกว่าชั้นนอกสุดของเยื่อบุผิวเหงือก จากนั้นการทดลองจึงเน้นในการใช้เนื้อเยื่อปริทันต์เป็นแบบจำลอง ข้อมูลการย้อมทางด้านอิมมูโนวิทยาแสดงให้เห็นอย่างสอดคล้องว่าพบการแสดงออกของโปรตีน MxA ในชั้นเยื่อบุผิวของเนื้อเยื่อปริทันต์ที่ปกติสูงกว่าเมื่อเทียบกับเนื้อเยื่อที่เป็นโรค ปริทันต์ โปรตีน MxA ของมนุษย์มีการคิดกันว่าถูกชักนำด้วย type I และ type III อินเตอร์เฟรอน แต่ผู้วิจัยไม่พบไซโตคายน์ชนิดนี้ในเนื้อเยื่อปริทันต์ที่ปกติ การกระตุ้นในหลอดทดลองของเซลล์เยื่อบุผิวจากเหงือกมนุษย์ด้วย แอลฟา-ดิเฟนซิน นำไปสู่การแสดงออกของโปรตีน MxA แต่ไม่พบปรากฏการณ์นี้ในเปปไทด์ต้านจุลชีพอื่น อย่าง เบต้า-ดิเฟนซิน หรือ LL37 แอลฟา-ดิเฟนซินยังถูกพบในเนื้อเยื่อปริทันต์ที่ปกติอีกด้วย ยิ่งไปกว่านี้ MxA ในเซลล์เยื่อบุผิวจากเหงือกมนุษย์ที่กระตุ้นด้วยแอลฟา-ดิเฟนซินมีความเกี่ยวข้องกับการป้องกันต่อการติดเชื้อไข้หวัดนก H5N1 และการ silencing ของยีน MxA ด้วย siRNA ที่จำเพาะต่อ MxA เลิกล้มความสามารถในการต้านไวรัสดังกล่าว เท่าที่เรารู้นี่เป็นการศึกษาแรกที่เปิดเผยถึงวิถีทางใหม่ของการชักนำการเกิด MxA ของมนุษย์ ซึ่งถูกเริ่มโดยเปปไทด์ต้านจุลชีพซึ่งเกิดขึ้นภายในช่องปาก ที่มีชื่อว่า แอลฟา-ดิเฟนซิน วิถีทางนี้อาจแสดงบทบาทสำคัญในการเป็นด่านแรกของการคุ้มกันต่อต้านไวรัสในเนื้อเยื่อปริทันต์

คำสำคัญ: แอลฟา-ดิเฟนซิน, MxA, ภาวะธำรงดุล, เนื้อเยื่อปริทันต์, การต้านไวรัส

หน้าสรุปโครงการ (Executive Summary)

1. **ชื่อโครงการ** ระบบภูมิคุ้มกันอินเนตในช่องปากที่มีต่อไวรัส

Innate antiviral immunity in the oral cavity

2. **ชื่อหัวหน้าโครงการ** รังสินี มหานนท์

Rangsini Mahanonda

ตำแหน่งทางวิชาการ รองศาสตราจารย์

หน่วยปฏิบัติการวิจัยโรคปริทันต์ : อิมมูโนวิทยา-พยาธิวิทยา / ปริทันต์บำบัด

คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ห้องปฏิบัติการอิมมูโนวิทยา คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ภาควิชา ปริทันตวิทยา คณะ ทันตแพทยศาสตร์

โทรศัพท์ 0-2218-8861 โทรสาร 0-2218-8851 E-mail : rangsini.m@chula.ac.th

3. **งบประมาณทั้งโครงการ** 1,999,900 บาท

4. **ระยะเวลาดำเนินงาน** 3 ปี

5. **ปัญหาที่ทำการวิจัยและความสำคัญของปัญหา**

Human mouth harbors millions of microbial organisms with remarkable diversity. These microorganisms include bacteria, viruses and fungi. We have demonstrated that cells of periodontal tissue such as gingival epithelium and fibroblasts are well equipped with pattern recognition receptors that are capable of recognizing oral bacteria as well as viruses and activating innate immune response (Mahanonda and Pichyangkul, 2007, Periodontology 2000; Mahanonda et al., 2009, J Periodont Res; Mahanonda et al., 2007, J Immunol). Innate immunity serves as the first line of defense against invading microorganisms. Interestingly, viral-infected oral/gingival lesions have been observed but generally the prevalence of those lesions is not so high. One of the critical questions that need to be addressed is how we live in harmony with many viruses in the oral cavity. The local innate immune response should be effective and keep viruses in check leading to oral homeostasis.

The study by our collaborator focusing on antiviral innate immune response in H5N1 infected lung demonstrated the presence of MxA expression in many cell types (Pichyangkul et al. submitted for publication, J Infect Dis). MxA is expressed in the cytoplasm and exhibits intrinsic antiviral activity. Interestingly, our preliminary observation showed the presence of cytoplasmic antiviral MxA protein in healthy periodontal tissue. So far there

has been no report of MxA expression in the oral cavity or other non-diseased tissue sites in the body. Therefore, we propose to evaluate the MxA and other antiviral protein expression in oral mucosa.

6. วัตถุประสงค์

1) Evaluate the expression of anti-viral proteins ; MxA, and other anti-viral molecules such as secretory leukocyte protease inhibitor (SLPI), protein kinase R (PKR), and OAS/RNASE L in healthy oral mucosa, periodontitis and OLP (N=10 from each group).
2) In vitro model: testing the effect of antimicrobial peptides (alpha and beta defensins, and cathelicidin), especially from PMN on anti-viral protein expression of primary oral epithelial cells.
3) The function of inducible anti-viral proteins in anti-microbial peptide-treated oral epithelial cells will be evaluated by protection using DNA virus (HSV) and RNA virus (H5N1) as a model.

7. ผลการดำเนินการวิจัย

1. We collected frozen tissue specimens from oral healthy biopsies, periodontitis biopsies and OLP.
2. We investigated the expression of anti-viral protein (human MxA) in oral mucosal section by immunohistochemical staining technique. We found that the human MxA protein expression was clearly observed in the epithelial layer of oral mucosa. Then the main experiments were focused on periodontal tissues. In periodontal tissue, intense MxA staining at the junctional epithelium was detected. Interestingly the level of MxA proteins in the epithelial layer was significantly higher in healthy periodontal tissues than in periodontitis and epithelial MxA protein was in close association with alpha-defensin-positive cells.
3. We evaluated the mRNA expression of anti-viral proteins (MxA, PKR, OAS, SLPI) from both healthy periodontal tissue and periodontitis specimens. We found mRNA expression of anti-viral proteins (MxA, PKR, OAS, SLPI) in all examined periodontal tissues. These differences between healthy and periodontitis tissues were not statistically significant.
4. We evaluated the effect of antimicrobial peptides on anti-viral protein (MxA) expression of primary oral epithelial cells. We clearly observed MxA protein expression after treatment with α-defensins-1, 2, or 3, but not with the other antimicrobial peptides β-defensins-1, 2, 3, or LL37.
5. We evaluated the function of inducible MxA protein in α-defensin-treated oral epithelial cells by H5N1 model. We demonstrated that MxA expressed in α-defensin-treated oral epithelial cells inhibited H5N1 viral replication. After silencing the MxA gene, oral epithelial cells treated with α-defensin-1 robustly down-regulated MxA function, allowing viral replication and cell death to occur.

6. We evaluated the function of inducible MxA protein in α-defensin-treated oral epithelial cells by HSV-1 model. We demonstrated that MxA expressed in α-defensin-treated oral epithelial cells did not inhibited cytopathic effect of HSV-1. However, we encountered technical problems and were not able to consistently demonstrated the amount of produced virus in culture supernatants. This experiment was then excluded from the study project.

8. แผนการดำเนินการวิจัยตลอดโครงการ

Year plan	1st year		2nd year		3rd year	
	1-6	7-12	13-18	19-24	25-30	31-36
1. Collect frozen tissue specimens form oral mucosal biopsies (Healthy, Periodontitis, OLP)	←	→				
2. Establish immunohistochemical staining technique	←	→				
3. Establish RT-PCR technique	←	→				
4. Collect healthy gingival biopsies and establish human primary gingival epithelial cell lines	←	→	→	→	→	→
5. Immunohistichemical staining and mRNA expression by RT-PCR to detect antiviral proteins			←	→		
6. Investigate the effect of antimicrobial peptides on antiviral protein expression			←	→		
7. Test function of inducible antiviral proteins in oral epithelial cells by measuring protection against herpes simplex virus and H5N1 (Dept. Microbiology, Faculty of Science, Mahidol Uni.)					←	→
8. Report and publication						↔

9. Publication

1. **Mahanonda R**, Sa-Ard-Iam N, Rerkyen P, Champaiboon C, Vanavit N, Pichyangkul S. Innate antiviral immunity of periodontal tissue. Periodontol 2000. 2011; 56(1): 143-53. Impact factor = 4.012 (ที่มา : Journal Citation Reports, 2012)
2. **Mahanonda R**, Sa-Ard-Iam N, Rerkyen P, Thitithanyanont A, Subbalekha K, Pichyangkul S. MxA expression induced by a-defensin in healthy periodontal tissue. Eur J Immunol. 2012; 42(4):946-956. Impact factor = 4.97 (ที่มา : Journal Citation Reports, 2012)
3. Thitithanyanont A, Engering A, Uiprasertkul M, Ekchariyawat P, Wiboon-Ut S, Kraivong R, Limsalakpetch A, Kum-Arb U, Yongvanitchit K, Sa-Ard-Iam N, Rukyen P, **Mahanonda R**, Kawkitinarong K, Auewarakul P, Utaisincharoen P, Sirisinha S, Mason CJ, Fukuda MM, Pichyangkul S. Antiviral immune responses in H5N1-infected human lung tissue and possible mechanisms underlying the hyperproduction of interferon-inducible protein IP-10. Biochem Biophys Res Commun. 2010; 6;398(4):752-8. Impact factor = 2.406 (ที่มา : Journal Citation Reports, 2012)
4. Pichyangkul S, Krasaesub S, Jongkaewwattana A, Thitithanyanont A, Wiboon-ut S, Yongvanitchit K, Limsalakpetch A, Kum-Arb U, Mongkolsirichaikul D, Khemmu N, **Mahanonda R**, Garcia JM, Mason CJ, Walsh DS, Saunders DL. Short Report: Pre-Existing Cross-Reactive Antibodies to Avian Influenza H5N1 and 2009 Pandemic H1N1 in US Military Personnel. Am J Trop Med Hyg. 2013. (Inpress) Impact factor = 2.534 (ที่มา : Journal Citation Reports, 2012)

Introduction

Viruses in the oral cavity

The human mouth harbors millions of highly diverse microbial organisms. These include approximately 700 species of bacteria, as well as viruses, fungi and protozoa (Dahlen, 2009; Paster et al., 2006). These microbes nearly outnumber the mucosal lining cells. They continually live and grow in an oral ecosystem which provides moisture, warmth and nutrients. Even though the oral mucosa is constantly exposed to a variety of microbes, most individuals maintain homeostasis, suggesting an effective innate immune system in the oral cavity.

The etiologic importance of bacteria in periodontal disease has been understood for decades. Bacterial plaque biofilms continually form on the tooth surfaces adjacent to gingiva. Gram-positive bacteria, aerobes and facultative anaerobes, including *Streptococcus*, *Veillonella* and *Actinomyces* species are a major presence in dental plaque and in the gingival sulcus of healthy and inflamed gingiva. Bacterial numbers are lower at healthy sites than inflamed ones. In contrast, most microbial plaques associated with periodontitis lesions are Gram-negative anaerobes and facultative anaerobes such as *Porphyromonas gingivalis*, *Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans* , and *Treponema denticola* (Haffajee and Socransky, 1994; Holt and Ebersole, 2005; Meyer and Fives-Taylor, 1997; Slots, 1999; Socransky et al., 1998).

Several studies have also documented a role of viruses in the development and progression of periodontitis and other oral infections. In particular, herpesviruses, a group of DNA viruses, have emerged as putative periodontal pathogens (Slots, 2005). Herpesviruses are commonly found in the oral cavity and most individuals become infected with herpesviruses in childhood. DNA from herpesviruses such as herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), human cytomegalovirus (HCMV), and Epstein-Barr virus (EBV) has been detected in subgingival plaque, gingival crevicular fluid (GCF), gingival tissue (Ehrlich, Cohen, and Hochman, 1983), and infiltrated immune cells from periodontitis sites (Contreras, Nowzari, and Slots, 2000; Contreras and Slots, 1996; Contreras et al., 1999; Parra and Slots, 1996; Saygun et al., 2002). Viral DNA can also be found at healthy sites, but generally at a lower frequency than at periodontitis sites (Contreras, Nowzari, and Slots, 2000; Contreras et al., 1999; Parra and Slots, 1996). Herpesvirus infection varies considerably in clinical outcome, ranging from subclinical to potentially lethal infections such as pneumonia, encephalitis and cancer, including carcinoma, sarcoma and lymphoma (Arduino and Porter, 2008; Mocarski, Shenk, and Pass, 2007; Pass, 2002; Roizman, Knipe, and Whitley, 2007; Slots, 2005; Williams and Crawford, 2006). Most human adults are carriers of HCMV and EBV. Both of these have been frequently associated with periodontal diseases such as chronic periodontitis (Saygun et al., 2002), aggressive periodontitis (Kubar et al., 2004; Yapar et al.,

2003), apical periodontitis lesions (Saboia-Dantas et al., 2007), and periodontal abscess (Saygun et al., 2004). mRNA transcription of HCMV has been demonstrated in deep periodontal pockets of chronic periodontitis and in localized juvenile periodontitis, but not in shallow periodontal sites, suggesting HCMV activation in periodontitis (Contreras and Slots, 1998; Ting, Contreras, and Slots, 2000).

Other viruses such as human papilloma virus (Madinier et al., 1992; Parra and Slots, 1996), human immunodeficiency virus (HIV), and hepatitis C virus (Maticic et al., 2001) have also been identified at periodontal disease sites (Contreras and Slots, 1996; Parra and Slots, 1996). However, their role in periodontal disease is not yet understood, and requires further investigation.

Innate immunity in the oral cavity

Innate immunity serves as the first line of defense against invading microorganisms. It consists of non-cellular and cellular components. Innate antibacterial immune response in the oral cavity has been the focus of considerable recent research (Schenkein, 2006; Teng, 2006; Zasloff, 2002). However, to date there is relatively little available information regarding oral innate antiviral immunity.

Oral mucosa

The oral mucosa forms an effective physical barrier. Many thick, multilayered epithelia seem to tolerate the attachment of microbes on the dead superficial epithelial layers which are continuously sloughed (Skougaard, 1970). Keratin, which coats most of the oral mucosa, provides a barrier against viral infection (Friedman, 2006). Additionally, oral epithelial cells express pattern recognition receptors (PRRs) that detect early viral invasion, leading to production of mediators that terminate viral infection.

As reviewed by Mahanonda and Pichyangkul (2007), cells of periodontal tissues (gingival cells, periodontal ligament fibroblasts, cementoblasts, osteoblasts) express a variety of TLRs. Primary human gingival epithelial cells (HGEC) derived from healthy periodontal tissues express mRNA of TLRs 1, 2, 3, 4, 5, 6, and 9, but not of TLRs 7 or 8 (Mahanonda et al., 2009). After TLR3 triggering by polyinosinic-polycytidylic acid (Poly I:C, a synthetic analog of dsRNA), HGECs express and secrete HBD-2 and IL-8, suggesting functional TLR3 (Mahanonda et al., 2009). Poly I:C also induces HGEC antiviral response via IFN-β production (unpublished data). On the other hand, TLR7 and 8 ligands are not able to induce HBD-2 or IL-8 expression, which confirms the absence of TLR7 and 8 expression in HGECs. Interestingly, HGECs clearly express TLR9 but do not respond to the TLR9 ligand-CpG ODN (a synthetic oligodeoxynucleotide) in *in vitro* cell cultures (Mahanonda et al., 2009). The reason for a nonfunctional TLR9 in HGECs remains unclear and requires further investigation.

The gingival sulcus: a strategic area in periodontal innate immunity

The gingival sulcus is a strategically important location where periodontal tissue is constantly challenged by microbial biofilms and their products. In healthy gingiva, the gingival sulcus is a shallow (approximately 0.69 mm deep), v-shaped crevice (Gargiulo, Wentz, and Orban, 1961). It is bounded by the tooth surface on one side and the sulcular epithelium on the other. At the bottom lies the junctional epithelium. The integrity of the junctional epithelium and the sulcular epithelium is crucial for maintaining periodontal health. The conversion of the junctional epithelium to pocket epithelium is widely regarded as a hallmark for the loss of periodontal attachment and the development of periodontitis (Bosshardt and Lang, 2005).

Unlike the conventional stratified squamous keratinzing epithelium lining the outer gingival aspect (oral epithelium), both the sulcular and junctional epithelium are non-keratinizing and thin (Carranza 's Clinical Periodontology 10th edition), and are apparently more susceptible to infection. Hence, efficient innate mechanisms against microbial invasion at such a sensitive area are essential. Research in recent years has shown that the junctional epithelium may play a much more active role in innate immunity than previously assumed. Junctional epithelial cells attach to each other only loosely, due to a low density of desmosomes and the lack of E-cadherin. Thus, the junctional epithelial intercellular space is considerably more permeable than that of the sulcular and oral epithelia (Bosshardt and Lang, 2005). Adhesion molecule-intracellular adhesion molecule-1 (Crawford and Hopp, 1990) and a potent chemokine for PMN, IL-8, (Tonetti, 1997) are expressed in the junctional epithelium. Expression is especially high in its superficial layer (Tonetti, Imboden, and Lang, 1998). This expression and the permeability of the junctional epithelium contribute to the observed constant recruitment and migration of PMNs from subepithelial connective tissue blood vessels through the junctional epithelium into the gingival sulcus (Tonetti, Imboden, and Lang, 1998). During migration, activated PMNs release HNPs, which in turn induce the release of epithelial IL-8 (Sakamoto et al., 2005; Van Wetering et al., 1997), thus providing a local chemokine network. In human GCF the estimated concentration of PMN is about 800,000 per ml (Skapski and Lehner, 1976). These migratory PMNs limit the growth and accumulation of dental plaque. The dynamic sequence described above results from crosstalk between resident non-immune cells and professional phagocytic cells, all of which participate in the local innate immune activation.

The GCF occurs in minute amounts and is a complex mixture of substances derived from serum, leukocytes, structural cells of the periodontium and plaque bacteria. Detailed analysis show the presence of serum albumin (Pisano et al., 2005), lactoferrin (Friedman, Mandel, and Herrera, 1983), lysozyme (Friedman, Mandel, and Herrera, 1983), HNPs (Pisano et al., 2005), HBDs, LL37, SLPI (Into et al., 2006), cystatin A (Pisano et al., 2005), statherin (Pisano et al., 2005), and other unidentified components (Pisano et al., 2005). In periodontal health, the presence of HNPs, HBDs, LL37, SLPI, lactoferrin, and cystatin in GCF suggests an ongoing innate

antibacterial and antiviral process. HNPs are produced by PMNs (Ganz, 1987; Rice et al., 1987). LL-37 are produced by both PMNs and epithelial cells (Zanetti, 2004), and HBDs are produced only by epithelial cells (Ganz, 2003).

The oral mucosal epithelium seems to be naturally resistant to viral infection. Recent reports document constitutive expression of mucosal antiviral protein-SLPI in oral epithelial cells of both healthy (Jana, Gray, and Shugars, 2005), and inflamed periodontal tissues (Into et al., 2006). Levels of SLPI significantly increase after *in vitro* exposure of gingival epithelial cells to HIV (Jana, Gray, and Shugars, 2005). Our preliminary data demonstrated myxovirus resistance protein A (MxA protein) expression in healthy gingival tissue (Figure 2).This protein, a product of the human orthomyxovirus resistance gene (*MX1* gene), is induced by type I interferon and plays a critical role in the immune responses against different viruses (Haller, Kochs, and Weber, 2007; Ronni et al., 1993). MxA is expressed in the cytoplasm and exhibits intrinsic anti-viral activity. So far there has been no report of MxA expression in the oral cavity or other non-diseased tissue sites in the body.

As mentioned earlier, several viruses have been detected in oral cavity and some have been described to be involved in periodontitis and OLP. To date there has been very limited information reported on the role of oral mucosa in anti-viral defense. We hypothesize that epithelial cells, the first cell barrier possess protective innate immunity against viruses. Loss of anti-viral defense may subsequently lead to susceptibility of viral infection in the oral cavity

References

Carranza 's Clinical Periodontology 10th edition.

Arduino, P. G., and Porter, S. R. (2008). Herpes Simplex Virus Type 1 infection: overview on relevant clinico-pathological features. *J Oral Pathol Med* 37; 107-21.

Bosshardt, D. D., and Lang, N. P. (2005). The junctional epithelium: from health to disease. *J Dent Res* 84: 9-20.

Contreras, A., Nowzari, H., and Slots, J. (2000). Herpesviruses in periodontal pocket and gingival tissue specimens. *Oral Microbiol Immunol* 15: 15-8.

Contreras, A., and Slots, J. (1996). Mammalian viruses in human periodontitis. *Oral Microbiol Immunol* 11: 381-6.

Contreras, A., and Slots, J. (1998). Active cytomegalovirus infection in human periodontitis. *Oral Microbiol Immunol* 13: 225-30.

Contreras, A., Zadeh, H. H., Nowzari, H., and Slots, J. (1999). Herpesvirus infection of inflammatory cells in human periodontitis. *Oral Microbiol Immunol* 14: 206-12.

Crawford, J. M., and Hopp, B. (1990). Junctional epithelium expresses the intercellular adhesion molecule ICAM-1. *J Periodontal Res* 25: 254-6.

Dahlen, G. (2009). Non-odontogenic infections in dentistry. *Periodontol 2000* 49: 7-12.

Ehrlich, J., Cohen, G. H., and Hochman, N. (1983). Specific herpes simplex virus antigen in human gingiva. *J Periodontol* 54: 357-60.

Friedman, H. M. (2006). Keratin, a dual role in herpes simplex virus pathogenesis. *J Clin Virol* 35: 103-5.

Friedman, S. A., Mandel, I. D., and Herrera, M. S. (1983). Lysozyme and lactoferrin quantitation in the crevicular fluid. *J Periodontol* 54: 347-350.

Ganz, T. (1987). Extracellular release of antimicrobial defensins by human polymorphonuclear leukocytes. *Infect Immun* 55: 568-71.

Ganz, T. (2003). Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol* 3: 710-20.

Gargiulo, A., Wentz, F., and Orban, B. (1961). Dimensions and relations of the dento-gingival junction in humans. *j Periodontol* 32: 261-7.

Haffajee, A. D., and Socransky, S. S. (1994). Microbial etiological agents of destructive periodontal diseases. *Periodontol 2000* 5: 78-111.

Haller, O., Kochs, G., and Weber, F. (2007). Interferon, Mx, and viral countermeasures. *Cytokine Growth Factor Rev* 18: 425-33.

Holt, S. C., and Ebersole, J. L. (2005). Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia: the "red complex", a prototype polybacterial pathogenic consortium in periodontitis. *Periodontol 2000* 38: 72-122.

Into, T., Inomata, M., Kanno, Y., Matsuyama, T., Machigashira, M., Izumi, Y., Imamura, T., Nakashima, M., Noguchi, T., and Matsushita, K. (2006). Arginine-specific gingipains from Porphyromonas gingivalis deprive protective functions of secretory leucocyte protease inhibitor in periodontal tissue. *Clin Exp Immunol* 145: 545-54.

Jana, N. K., Gray, L. R., and Shugars, D. C. (2005). Human immunodeficiency virus type 1 stimulates the expression and production of secretory leukocyte protease inhibitor (SLPI) in oral epithelial cells: a role for SLPI in innate mucosal immunity. *J Virol* 79: 6432-40.

Kubar, A., Saygun, I., Yapar, M., Ozdemir, A., and Slots, J. (2004). Real-time PCR quantification of cytomegalovirus in aggressive periodontitis lesions using TaqMan technology. *J Periodontal Res* 39: 81-6.

Madinier, I., Doglio, A., Cagnon, L., Lefebvre, J. C., and Monteil, R. A. (1992). Southern blot detection of human papillomaviruses (HPVs) DNA sequences in gingival tissues. *J Periodontol* 63; 667-73.

Mahanonda, R., and Pichyangkul, S. (2007). Toll-like receptors and their role in periodontal health and disease. *Periodontol 2000* 43: 41-55.

Mahanonda, R., Sa-Ard-Iam, N., Eksomtramate, M., Rerkyen, P., Phairat, B., Schaecher, K. E., Fukuda, M. M., and Pichyangkul, S. (2009). Cigarette smoke extract modulates human beta-defensin-2 and interleukin-8 expression in human gingival epithelial cells. *J Periodontal Res* 44,: 557-64.

Maticic, M., Poljak, M., Kramar, B., Seme, K., Brinovec, V., Meglic-Volkar, J., Zakotnik, B., and Skaleric, U. (2001). Detection of hepatitis C virus RNA from gingival crevicular fluid and its relation to virus presence in saliva. *J Periodontol* 72: 11-6.

Meyer, D. H., and Fives-Taylor, P. M. (1997). The role of Actinobacillus actinomycetemcomitans in the pathogenesis of periodontal disease. *Trends Microbiol* 5; 224-8.

Mocarski, E. J., Shenk, T., and Pass, R. (2007). Cytomegaloviruses. In: Knipe, DM., Howley, PM., editors. *Fields Virology* 5th edn.(Philadelphia: Lipponcott, Williams & Wilkins), 2702-2772.

Parra, B., and Slots, J. (1996). Detection of human viruses in periodontal pockets using polymerase chain reaction. *Oral Microbiol Immunol* 11: 289-93.

Pass, R. F. (2002). Cytomegalovirus infection. *Pediatr Rev* 23: 163-70.

Paster, B. J., Olsen, I., Aas, J. A., and Dewhirst, F. E. (2006). The breadth of bacterial diversity in the human periodontal pocket and other oral sites. *Periodontol 2000* 42: 80-7.

Pisano, E., Cabras, T., Montaldo, C., Piras, V., Inzitari, R., Olmi, C., Castagnola, M., and Messana, I. (2005). Peptides of human gingival crevicular fluid determined by HPLC-ESI-MS. *Eur J Oral Sci* 113: 462-8.

Rice, W. G., Ganz, T., Kinkade, J. M., Jr., Selsted, M. E., Lehrer, R. I., and Parmley, R. T. (1987). Defensin-rich dense granules of human neutrophils. *Blood* 70: 757-65.

Roizman, B., Knipe, D. M., and Whitley, R. (2007). Herpes simplex viruses. In: Knipe DM, Howley PM, editors. *Fields Virology* 5th edn(Philadelphia: Lippincott, Williams & Wilkins), 2502-2601.

Ronni, T., Melen, K., Malygin, A., and Julkunen, I. (1993). Control of IFN-inducible MxA gene expression in human cells. *J Immunol* 150: 1715-26.

Saboia-Dantas, C. J., Coutrin de Toledo, L. F., Sampaio-Filho, H. R., and Siqueira, J. F., Jr. (2007). Herpesviruses in asymptomatic apical periodontitis lesions: an immunohistochemical approach. *Oral Microbiol Immunol* 22: 320-5.

Sakamoto, N., Mukae, H., Fujii, T., Ishii, H., Yoshioka, S., Kakugawa, T., Sugiyama, K., Mizuta, Y., Kadota, J., Nakazato, M., and Kohno, S. (2005). Differential effects of alpha- and beta-defensin on cytokine production by cultured human bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 288: L508-13.

Saygun, I., Sahin, S., Ozdemir, A., Kurtis, B., Yapar, M., Kubar, A., and Ozcan, G. (2002). Detection of human viruses in patients with chronic periodontitis and the relationship between viruses and clinical parameters. *J Periodontol* 73: 1437-43.

Saygun, I., Yapar, M., Ozdemir, A., Kubar, A., and Slots, J. (2004). Human cytomegalovirus and Epstein-Barr virus type 1 in periodontal abscesses. *Oral Microbiol Immunol* 19: 83-7.

Schenkein, H. A. (2006). Host responses in maintaining periodontal health and determining periodontal disease. *Periodontol 2000* 40: 77-93.

Skapski, H., and Lehner, T. (1976). A crevicular washing method for investigating immune components of crevicular fluid in man. *J Periodontal Res* 11: 19-24.

Skougaard, M. R. (1970). Cell renewal, with special reference to the gingival epithelium. *Adv Oral Biol* 4: 261-88.

Slots, J. (1999). Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis in periodontal disease: introduction. *Periodontol 2000* 20: 7-13.

Slots, J. (2005). Herpesviruses in periodontal diseases. *Periodontol 2000* 38: 33-62.

Socransky, S. S., Haffajee, A. D., Cugini, M. A., Smith, C., and Kent, R. L., Jr. (1998). Microbial complexes in subgingival plaque. *J Clin Periodontol* 25: 134-44.

Teng, Y. T. (2006). Protective and destructive immunity in the periodontium: Part 1--innate and humoral immunity and the periodontium. *J Dent Res* 85: 198-208.

Ting, M., Contreras, A., and Slots, J. (2000). Herpesvirus in localized juvenile periodontitis. *J Periodontal Res* 35: 17-25.

Tonetti, M. S. (1997). Molecular factors associated with compartmentalization of gingival immune responses and transepithelial neutrophil migration. *J Periodontal Res* 32: 104-9.

Tonetti, M. S., Imboden, M. A., and Lang, N. P. (1998). Neutrophil migration into the gingival sulcus is associated with transepithelial gradients of interleukin-8 and ICAM-1. *J Periodontol* 69: 1139-47.

Van Wetering, S., Mannesse-Lazeroms, S. P., Van Sterkenburg, M. A., Daha, M. R., Dijkman, J. H., and Hiemstra, P. S. (1997). Effect of defensins on interleukin-8 synthesis in airway epithelial cells. *Am J Physiol* 272: L888-96.

Williams, H., and Crawford, D. H. (2006). Epstein-Barr virus: the impact of scientific advances on clinical practice. *Blood* 107: 862-9.

Yapar, M., Saygun, I., Ozdemir, A., Kubar, A., and Sahin, S. (2003). Prevalence of human herpesviruses in patients with aggressive periodontitis. *J Periodontol* 74: 1634-40.

Zanetti, M. (2004). Cathelicidins, multifunctional peptides of the innate immunity. *J Leukoc Biol* 75: 39-48.

Zasloff, M. (2002). Innate immunity, antimicrobial peptides, and protection of the oral cavity. *Lancet* 360: 1116-7.

Materials and Methods

Reagents

Antimicrobial peptides: human α-defensins 1, 2, and 3, human β-defensins 1, 2, and 3, and LL37 were obtained from Innovagen. IFN-α and neutralizing antibodies against IFN-α and IFN-β were purchased from PBL Biomedical Laboratory. Neutalizing antibody against α-defensins was obtained from Hycult biotech (Uden, The Netherlands). Polymorphprep was purchased from Axis-Shield PoC AS (Oslo, Norway).

Human periodontal tissue sample collection

Tissue specimens were collected from patients (one biopsy per one patient) at Periodontal Clinic and Department of Oral Maxillofacial Surgery, Faculty of Dentistry, Chulalongkorn University. The ethical approval by the ethics committee of Faculty of Dentistry, Chulalongkorn University and informed consent of all participating subjects were obtained before operation. Healthy periodontal tissue samples were collected from sites with clinically healthy gingiva (no gingival inflammation, probing depth < 4 mm, and no radiographic bone loss) during crown-lengthening procedure for prosthetic reasons. Severe periodontitis tissue samples were collected from sites of extracted teeth with hopeless prognosis (inflamed gingiva, probing depth > 6 mm, and bone loss > 60% of the root).

Periodontal tissue specimens used for immunostaining, real-time quantitative RT-PCR, and *in vitro* cultures were derived from different donors.

Human gingival epithelial cells

The primary HGECs, derived from healthy periodontal tissue, were obtained following established procedure (Mahanonda et al., 2009. In brief, the excised tissues were immediately washed with Dulbecco's phosphate buffered saline and digested in 0.2% dispase for 24 h at 4°C. The separated epithelial layer was washed, minced and cultured in a serum-free keratinocyte growth medium (Clonetics) supplemented with human recombinant epidermal growth factor, hydrocortisone, bovine insulin, bovine pituitary extract, gentamicin sulfate, amphotericin B, and 0.15 mM $CaCl_2$. The HGEC cultures at passage two to four were used throughout the study.

Real-time RT-PCR

Total RNA from periodontal tissue samples and HGECs were isolated by using an RNeasy Mini kit from Qiagen. One microgram of DNase I-treated total RNA were reverse transcribed using ImProm-II Reverse Transcription System for RT-PCR (Promega). The cDNA was then divided and used for PCR amplification of

antiviral protein and cytokine expression. Real-time RT-PCR assays were performed on LightCycler System 480 (Roche Molecular Diagnostics) using SYBR Green PCR Master Mix (Roche Molecular Diagnostics). MxA, PKR, OAS, SLPI, IFN-α, IFN-β, IFN-λ, and GAPDH were amplified using specific primers purchased from Operon. The primer sequences are shown below.

MxA (5'-Gctacacaccgtgacggatatgg-3'/5'-CGAGCTGGATTGGAAAGCCC-3'),

PKR (5'- GCCTTTTCATCCAAATGGAATTC-3'/5'-GAAATCTGTTCTGGGCTCATG -3'),

OAS (5'-CATCCGCCTAGTCAAGCACTG-3'/5'-CCACCACCCAAGTTTCCTGTAG-3'),

SLPI (5'-TTCCCCTGTGAAAGCTTGATTC-3'/5'-GATATCAGTGGTGGAGCCAAGTC-3'),

IFN-α (5'-GGATGAGACCCTCCTAGACAAAT-3'/5'-ATGATTTCTGCTCTGACAACCTC-3'),

IFN-β (5'-GATTCATCTAGCACTGGCTGG-3'/5'-CTTCAGGTAATGCAGAATCC-3'),

IFN-λ (5'-GGACGCCTTGGAAGAGTCACT-3'/5'-AGAAGCCTCAGGTCCCAATTC-3'),

GAPDH (5'-GAAGGCTGGGGCTCATTT-3'/5'-CAGGAGGCATTGCTGATGAT-3').

Amplification conditions, sequences, and concentrations of the primers were similar to those of RT-PCR. After 45 reaction cycles, the melting curve analysis was performed at 95°C for 5 s, 65°C for 1 min, and heating to 97°C using a ramp rate of 0.11°C/sec with continuous monitoring of fluorescence. The melting peak generated represented the specific amplified product. All samples had only a single peak, indicating a pure product and no primer/dimer formation. Amplicons of a single band with the expected sizes were also confirmed in all reactions by agarose gel electrophoresis. The amplification efficiencies were high (close to 100%) when multiple standard curves were performed using serial mRNA dilutions.

For periodontal tissue specimens, the relative mRNA expression of antiviral proteins and cytokines was normalized to corresponding GAPDH for each sample, using the formula= $2^{-\Delta CT}$, where $\Delta CT = C_{T\text{-}geneX} - C_{T\text{-}GAPDH}$. The relative quantification of mRNA expression in periodontitis tissues was presented as the mean fold increase ± SEM, using the mean value obtained from the healthy tissue as a reference (relative quantification = 1).

For HGEC culture, fold differences in mRNA expression levels of antiviral proteins and cytokines between sample A and sample B was calculated using the $\Delta\Delta C_T$ method [47]. Levels of gene of interest were normalized to corresponding GAPDH for each sample, and the fold increase between sample A and sample B was calculated as follows: Fold increase = $2^{-\Delta\Delta CT}$, where

$-\Delta\Delta C_T = (C_{T\text{-}geneX} - C_{T\text{-}GAPDH})_{sample\ A} - (C_{T\text{-}gene\ X} - C_{T\text{-}GAPDH})_{sample\ B}$

Immunohistochemical analysis of MxA protein and α-defensins in periodontal tissue

The excised periodontal tissues were immediately washed in normal saline solution, placed in the optimum cutting temperature embedding compound, snap-frozen in liquid nitrogen, and stored at -80°C. Single immunohistochemical staining was performed via Polymer/HRP and DAB+ chromagen system (DAKO EnVision™

G/2 Doublestain System) on the frozen sections. After fixation in acetone, they were stained with primary mouse-anti-human mAb against human-MxA (clone M143, Dr. Haller, University of Freiburg, Freiburg, Germany), human α-defensins or isotype control.

Three selective areas of oral epithelium: upper, middle, and lower parts of each tissue specimen were counted for MxA positive cells. The immunoreactivity of MxA staining was given a semiquantitative score ranging from score 1-3. Score 1 = the area of positive cells was less than 10% in the counting field, score 2= 10-50%, and score 3 = more than 50%.

Effects of antimicrobial peptides on HGECs

Non-toxic concentrations of different antimicrobial peptides for HGECs were predetermined as assessed by cell viability (MTT assay and Trypan blue exclusion). HGECs, normal human bronchial epithelial cells (Clonetics) and primary human microvascular endothelial cells (Clonetics) were treated with non-toxic doses of either α-defensin-1 (10 μM); α-defensin-2 (10 μM); α-defensin-3 (10 μM); β-defensin-1 (10 μM); β-defensin-2 (10 μM); β-defensin-3 (0.5 μM); LL37 (2 μg/ml); or IFN-α (100 U/ml). After 6 h of treatment with antimicrobial peptide or cytokine, mRNA expression of MxA was analyzed. In neutralization experiment, cells were treated with α-defensin-1 or IFN-α in the absence or presence of neutralizing antibodies against IFN-α (400 neutralization unit/ml) and IFN-β (400 neutralization unit/ml). After 24 h of treatment, immunohistochemical analysis of MxA protein was carried out.

Virus

H5N1 virus (A/open-billed stork/Nahkonsawan/BBD0104F/04) was isolated from cloacal swabs of live Asian open-billed storks between 2004 - 2005 and propagated in Madin-Darby canine kidney cells using MEM (Gibco) supplemented with 10% FBS (Hyclone) and penicillin and streptomycin (Thitithanyanont et al., 2007). The sequence data of the virus was submitted to GenBank with accession numbers DQ989958. The virus was grown in Madin-Darby canine kidney cells cells and the titer of virus stock was determined as described previously (Thitithanyanont et al., 2007). All experiments with H5N1 virus were performed in a Biosafety Level 3 facility (Mahidol University) by trained researchers.

Standard HSV-1 stain KOS will be a kind gift from Dr Parvapan Bhattarakosol, Department of Microbiology, Faculty of Medicine, Chulalongkorn University). H5N1 virus (A/open-billed stork/Nahkonsawan/BBD0104F/04) will be a kind gift from Dr Arunee Thitithanyanont, Department of Microbiology, Faculty of Science, Mahidol University).

HSV-1 is grown in African green monkey kidney (Vero) cells in DMEM (Gibco, USA) supplemented with 10% FBS (Hyclone) and antibiotic–antimycotic solution (Invitrogen). The stocks will be propagated in Vero cells

and stored at -80 °C at a concentration of 3.5×10^8 plaque forming units (PFU) and diluted in culture medium immediately before use.

H5N1-infected HGECs

HGECs (40,000 cells/well) were treated with either α-defensin-1 (10 μM); α-defensin-2 (10 μM); α-defensin-3 (10 μM); β-defensin-1 (10 μM); β-defensin-2 (10 μM); β-defensin-3 (0.5 μM); LL37 (2 μg/ml); or IFN-α (100 U/ml) for 24 h. They were washed 2 times and then co-cultured with H5N1 virus at MOI 1 (1 PFU/cell). After 1 h, the inoculum virus was removed and the HGECs were washed 2 times with PBS and cultured with fresh medium. Virus titers in culture supernatants and cytopathic effect were determined 48 h post-infection. To assess the number of infectious particles (plaque titers) in cell culture supernatants a plaque assay using Avicel (RC-591, FMC Biopolymer, Germany) was performed in 96-well plates Matrosovich et al., 2006; Rowe et al., 1999. Virus-infected cells were immunostained by incubating for 1 h with a monoclonal antibody specific for the influenza A virus nucleoprotein (Chemicon) followed by 30 min incubation with peroxidase-labeled anti-mouse antibody (Southern Biotech) and 30 min incubation with True blue™ peroxidase substrate (KPL). The PFU were counted with ELISPOT reader (Ziess, Germany). Percentage of H5N1 inhibition was then calculated.

Cell death reflecting cytopathic effect of H5N1 infection was observed under a microscope All experiments with H5N1 virus were performed in a Biosafety Level 3 facility.

HSV1-infected HGECs

HGECs (40,000 cells/well) were treated with either α-defensin-1 (10 μM); α-defensin-2 (10 μM); α-defensin-3 (10 μM); β-defensin-1 (10 μM); β-defensin-2 (10 μM); β-defensin-3 (0.5 μM); LL37 (2 μg/ml); or IFN-α (100 U/ml) for 24 h. They were washed 2 times and then co-cultured with HSV-1 virus at MOI 1 (1 PFU/cell). After 1 h, the inoculum virus was removed and the HGECs were washed 2 times with PBS and cultured with fresh medium. Cytopathic effect were determined 48 h post-infection under a microscope.

siRNA, transfection and infection

MxA siRNA was purchased form Santa Cruz Biotechnology. Briefly, semi-confluent HGECs were seeded with growth media without antibiotics one day before transfection. 80 nM MxA siRNA and 5 ul siRNA tranfection reagent were diluted in 1 ml of transfection medium, mixed and incubated at room temperature for 45 mins. HGECs were washed 2 times with transfection medium and then the dilutes MxA siRNA was added for 7 h, then 2x growth medium was added and cells were cultured overnight. Depletion of MxA expression by MxA siRNA was assessed by real-time RT-PCR and immunohistochemistry (for protein level). Transfected HGECs were treated with α-defensin-1 overnight and then infected with H5N1 virus.

PMN culture

Highly purified PMNs from healthy human subjects were prepared by density centrifugation using Polymorphprep™. The purity of PMNs was > 95%, as determined by anti-CD16 mAb using flow cytometry. PMNs (5×10^6 cells/ml) were incubated for 6 h in serum-free keratinocyte growth medium. Supernatants were collected for measurement of α-defensin production by ELISA (detected all human α-defensins-1, 2, and 3). PMN supernatants with or without neutralizing antibody against α-defensins (neutralizes all human α-defensins-1, 2, and 3; 1 μg/ml) or neutralizing antibodies against IFN-α (400 neutralization unit/ml) and IFN-β (400 neutralization unit/ml) was added to HGEC cultures. After 6 h of treatment with either PMN supernatant or medium control, mRNA expression of MxA was analyzed by real-time RT-PCR. After 24 h incubation, MxA protein expression in HGECs was analyzed by immunohistochemistry.

Statistical analysis

The parametric Student's *t*-test was used for normally distributed data, and the nonparametric Mann-Whitney rank-sum test was used for non-normally distributed data. A p-value < 0.05 was considered statistically significant. Data were analyzed with SPSS Version 11.5 software (SPSS Inc.).

References

Mahanonda, R., Sa-Ard-Iam, N., Eksomtramate, M., Rerkyen, P., Phairat, B., Schaecher, K. E., Fukuda, M. M. and Pichyangkul, S. (2009) Cigarette smoke extract modulates human beta-defensin-2 and interleukin-8 expression in human gingival epithelial cells. *J Periodontal Res* 44: 557-564.

Matrosovich, M., Matrosovich, T., Garten, W. and Klenk, H. D. (2006) New low-viscosity overlay medium for viral plaque assays. *Virol J* 3: 63.

Rowe, T., Abernathy, R. A., Hu-Primmer, J., Thompson, W. W., Lu, X., Lim, W., Fukuda, K. *et al*., (1999) Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. *J Clin Microbiol* 37: 937-943.

Thitithanyanont, A., Engering, A., Ekchariyawat, P., Wiboon-ut, S., Limsalakpetch, A., Yongvanitchit, K., Kum-Arb, U. *et al*., (2007) High susceptibility of human dendritic cells to avian influenza H5N1 virus infection and protection by IFN-alpha and TLR ligands. *J Immunol* 179: 5220-5227.

Results

MxA protein expression in periodontal tissues

MxA protein is well known to have antiviral activity against both RNA and DNA viruses Netherton el al., 2009; Pavlovic, Haller and Staeheli 1992. We focused on MxA protein throughout our study. Immunohistochemical data in Fig. 1*A* and 1*B* shows that MxA protein expression was clearly observed in the epithelial layer of periodontal tissue. Whereas Fig 1C and 1D shows that MxA protein expression was minimally observed in the epithelial layer of normal buccal mucosa and oral lichen planus. Epithelial MxA immunoreactivity seemed to be stronger in basal and spinous layers than outermost layer of oral epithelium. Then the main experiments were focused on periodontal tissues.

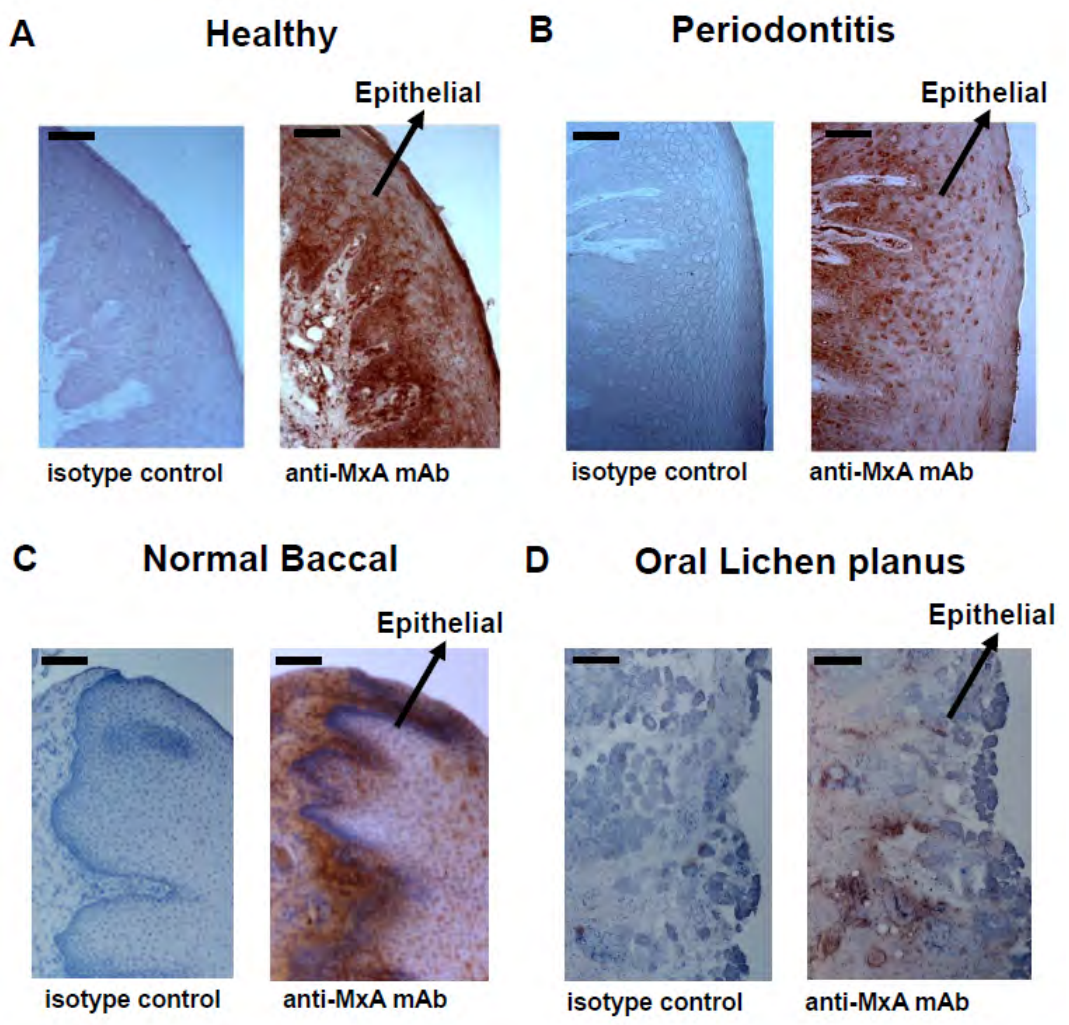

Figure 1. MxA protein expression in the epithelial layer and increased expression in healthy periodontal tissue. Frozen sections of (A) healthy periodontal tissues, (B) tissues with periodontitis, (C) healthy normal buccal tissues and (D) tissues with oral lichen planus were stained with either an anti-MxA antibody and isotype control by immunoperoxidase technique, and counterstained with haematoxylin. Data are representative of nine healthy periodontal tissues and seven periodontitis tissues. Each scale bar represents100 μM.

Using semiquantitative scoring, there was a significantly higher score of epithelial MxA in healthy group than periodontitis group (Table 1) (p= 0.012), thus highlighting the role of MxA protein in healthy periodontal tissue.

Group	Immunoreactive staining score			
	0 n (%)	1 n (%)	2 n (%)	3 n (%)
Healthy	0	0	0	9 (100)
Periodontitis	0	1 (14)	3 (43)	3 (43)

Score 1 = the area of positive cells < 10%, score 2 = 10–50%, and score 3 = > 50%.

Table 1: Immunoreactive staining score of MxA protein in epithelial layer of healthy (n = 9) and periodontitis (n = 7) tissue specimens.

mRNA expression of antiviral proteins in periodontal tissues

SLPI has been reported in relation to antiviral defense in periodontal tissue Jana, Gray and Shugars 2005. In this study we evaluated the expression of other antiviral molecules, including MxA, OAS, and PKR from both healthy periodontal tissue and periodontitis specimens. Using real-time RT-PCR, we found mRNA expression of MxA, OAS, PKR, and SLPI in all examined periodontal tissues. As compared with healthy periodontal tissues, the mean fold increase of relative quantification of MxA, OAS, PKR, and SLPI in periodontitis tissues was 0.83 ± 0.24, 1.06 ± 0.30, 1.20 ± 0.34, and 2.74 ± 1.37 respectively (Fig. 2). These differences between healthy and periodontitis tissues were not statistically significant ($p>0.05$).

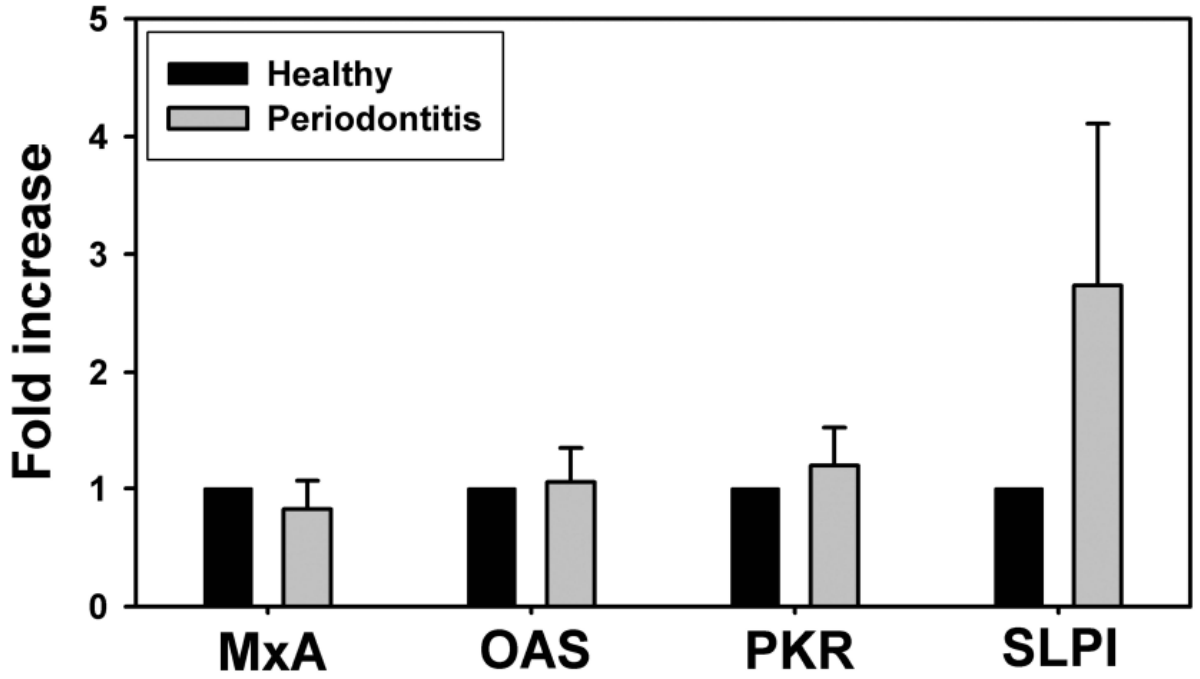

Figure 2. No differences in mRNA expression of antiviral proteins between healthy and periodontitis tissue. Periodontal tissue specimens were used for real-time RT-PCR for each of the indicated molecules as described in Materials and methods .The relative quantification of mRNA expression for each antiviral protein in the periodontitis tissues was normalized to corresponding GAPDH, and presented as the mean fold increase ± SEM, considering the mean value obtained from healthy periodontal tissues as a reference (relative quantification = 1) (n = 10 in each group).

α defensin-induced MxA protein expression in HGECs

Since MxA protein is known to be induced by type I and type III IFN (Haller, Frese and Kochs 1998; Brand et al., 2005; Holzinger et al., 2007). we then investigated the presence of type I and type III IFN in periodontal tissue. The mRNA expression of IFN-α, IFN-β, and IFN-λ in healthy periodontal tissue was negligible (*n* = 10, data not shown). The findings led us to hypothesize that other local mediators may be responsible for the observed MxA protein expression in healthy periodontal tissue.

Antimicrobial peptides including α-defensin, β-defensin and LL37 are constitutively expressed in healthy periodontal tissue (Diamond, Beckloff and Ryan 2008) and these mediators could conceivably play a role in MxA expression. Furthermore, a recent study described a fish homologue of MxA protein which was induced by human α-defensin (Falco et al., 2007). Therefore, we stimulated primary HGEC cultures with non-toxic concentrations of α-defensins-1, 2, and 3, β-defensins-1, 2, and 3, and LL37. Fig. 3A shows that α-defensins-1, 2,

and 3 markedly induced MxA protein in HGECs. There seemed to be stronger MxA staining in HGECs treated with α-defensin-1 than in those treated with α-defensin-2 and α-defensin-3. In contrast, β-defensins-1, 2, 3 and LL37 induced only negligible MxA protein expression. IFN-α was used as positive control and induced strong MxA protein expression. The results of MxA protein expression induced by α-defensins-1, 2, and 3, β-defensins-1, 2, and 3, and LL37 agree with mRNA expression using real-time RT-PCR (Fig. 3B).

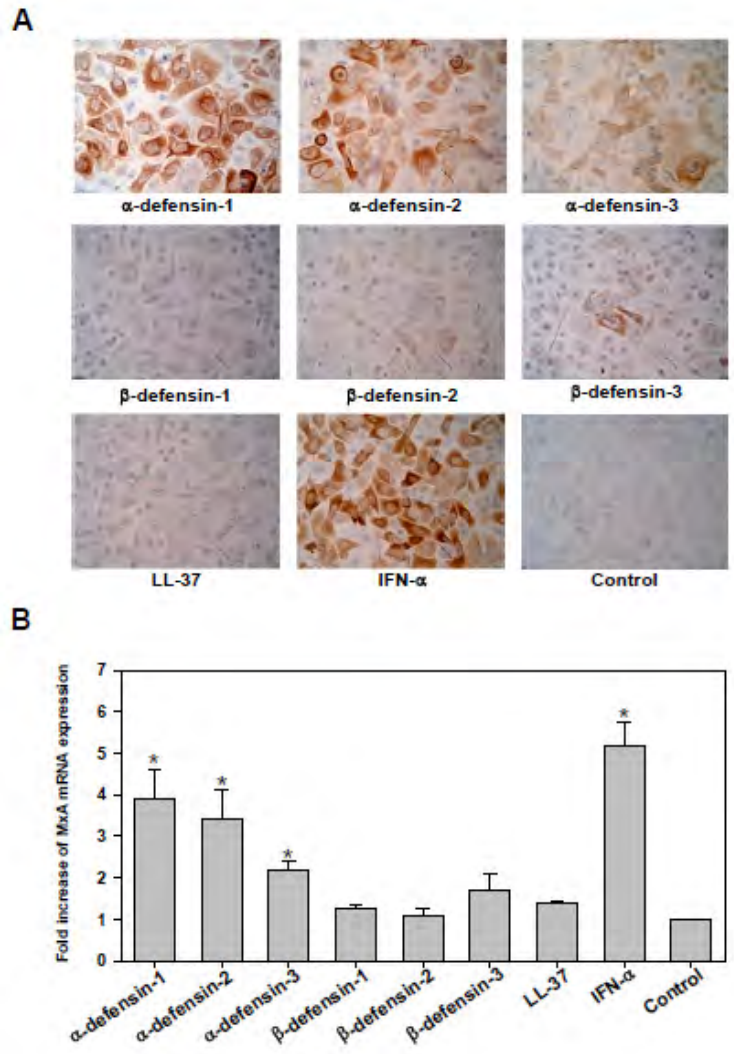

Figure 3. α-defensins induced protein and mRNA expression of MxA in HGECs and other cells. (A) HGECs were treated for 24 h with the indicated antimicrobial peptides as described in Materials and methods. IFN-α was used as a positive control and culture medium as a negative control. Immunohistochemical staining with anti-MxA antibody was used to detect MxA protein in treated HGECs. Data are representative of four separate experiments. One sample per experiment and each sample derived from different individual donors. (B) Real-time

RT-PCR was also used to analyze MxA mRNA expression in 6 h-treated HGECs. Data are demonstrated as the mean fold increase ± SEM of MxA mRNA expression compared with medium control after normalization with the GAPDH of four separate experiments. One sample per experiment and each sample derived from different individual donors (*, $p < 0.05$, compared with medium control).

α-defensin-1 was also able to stimulate MxA protein expression in other cells including normal human bronchial epithelial cells and primary human microvascular endothelial cells (Fig. 4).

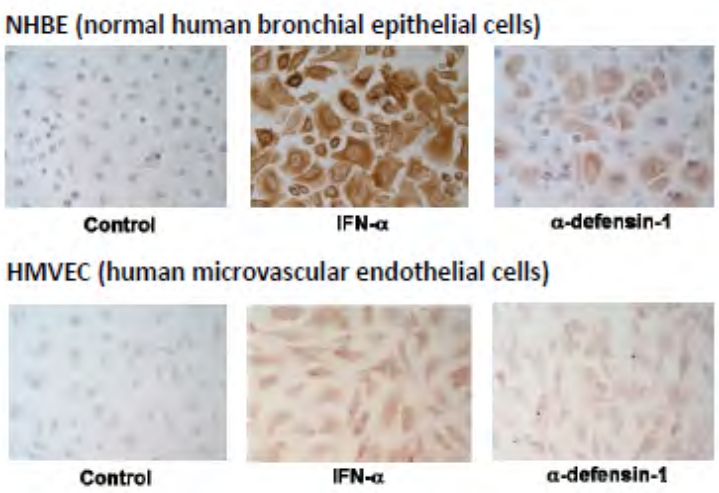

Figure 4. α-defensins induced protein of MxA in other cells. Normal human bronchial epithelial cells and primary human microvascular endothelial cells were treated for 24 h with α-defensin-1. Immunohistochemical staining with anti-MxA antibody was used to detect MxA protein in treated cells. IFN-α was used as positive control and culture medium was used as negative control. Data are representative of four separated experiments.

Addition of neutralizing antibodies against type I IFN (IFN-α and IFN-β) into the cultures of α-defensin-1-treated HGECs had no effect on MxA expression whereas neutralizing antibody against IFN-α markedly inhibited MxA expression in IFN-α-treated HGECs (Fig. 5).

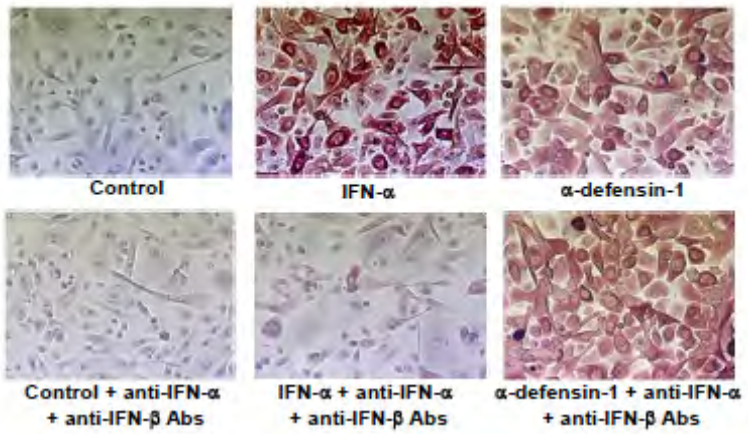

Figure 5. Neutralizing antibody against α-defensin-1 inhibited MxA expression. HGECs were treated for 24 h with: α-defensin-1 or IFN-α in the absence or presence of neutralizing antibodies against type I IFN (IFN-α and IFN-β). Culture medium was used as negative control. Immunohistochemical staining with anti-MxA antibody was used to detect MxA protein in treated HGECs. Data are representative of four separate experiments. One sample per experiment and each sample derived from different individual donors.

The IFN-α-induced MxA protein expression was likely to be independent on α-defensins since no detection of α-defensin production was observed in cultures of IFN-α-treated HGECs (Fig. 6). In addition, no production of type I IFN (IFN-α and IFN-β) was observed at both the mRNA and protein levels in α-defensin-treated HGECs (data not shown). Collectively, these data suggest that α-defensin and type I interferon use different triggering pathways to induce MxA expression.

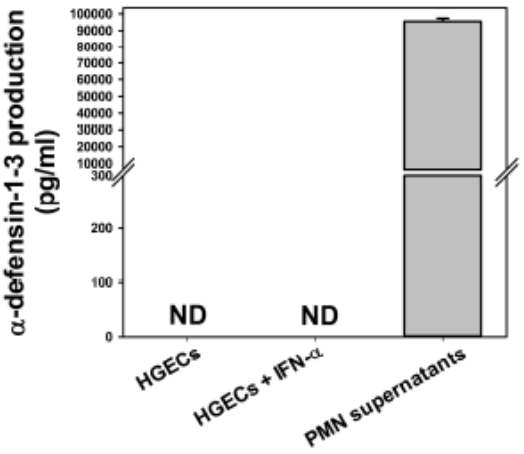

Figure 6. IFN-α does not induce α-defensin production from HGEC. Production of α-defensins was evaluated by ELISA. Culture medium was used as a negative control and PMN culture supernatants (6 h) were used as a positive control. Data are mean α-defensin levels ± SE of four separate experiments. ND = not detected.

The mechanism of α-defensin-1-induced MxA expression is different from IFN-α-induced MxA expression since we could not detect the enhanced expression of other ISGs including STAT1, STAT2, IRF3, IRF7, and IRF9 (Fig. 7).

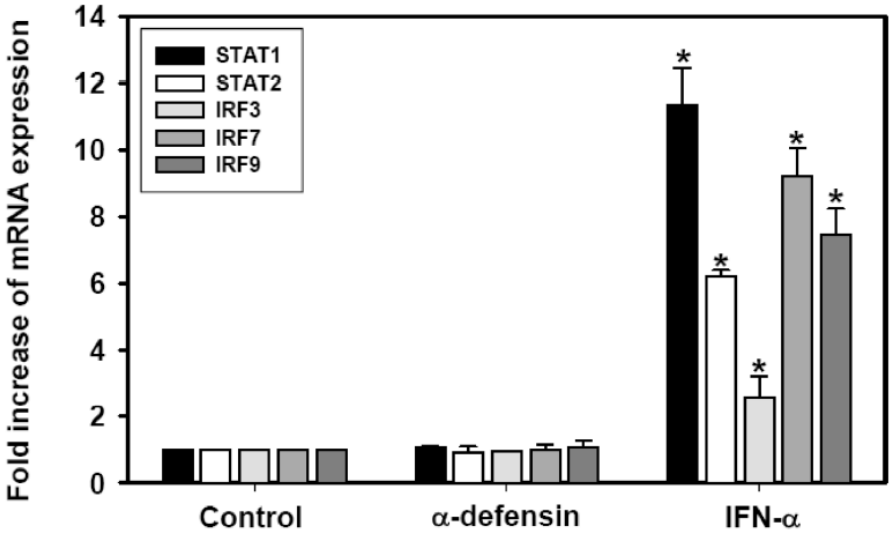

Figure 7. mRNA expression of STAT1, STAT2, IRF3, IRF7 and IRF9 in a-defensin-1-treated HGEC by real-time PCR. Data are demonstrated as the mwan fold increase± SE of STAT1, STAT2, IRF3, IRF7 and IRF9 mRNA expression compared with medium control after normalization with GAPDH of four separate experiment (*,

$p<0.05$, compared with control). IFN-a was used as positive control and culture medium was used as negative control.

Furthermore, STAT1 activation was also not detected in α-defensin-1-treated HGECs (Fig. 8).

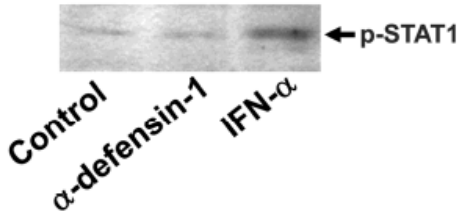

Figure 8. α -defensin-1 dose not induce STAT1 activation in HGECs. HGECS were treated with a-defensin-1. IFN-α was used as positive control and culture medium was used as negative control. The level of pY701-STAT1 in HGEC was determined by immunoblotting using specific antibody against pY701-STAT1. Data are representative of one form two separate experiments.

Role of periodontal tissue MxA against influenza A viral infection

The antiviral activity of MxA against influenza A virus is well recognized (Pavlovic, Haller and Staeheli 1992). Our preliminary data showed that unlike seasonal influenza virus, avian influenza H5N1 virus infected primary HGECs. We then employed H5N1 infection as a model to study the antiviral activity of α-defensin-induced MxA. The viral plaque assay in Fig. 9A shows that, similar to IFN-α-pretreated HGECs, α-defensins-1, 2, and 3-pretreated cells significantly inhibited H5N1 replication, suggesting a functional MxA protein. On the other hand, β-defensins-1, 2, 3, and LL37-pretreated HGECs poorly inhibited viral replication. These findings were confirmed by microscopically observed cytopathic effects (Fig. 9B).

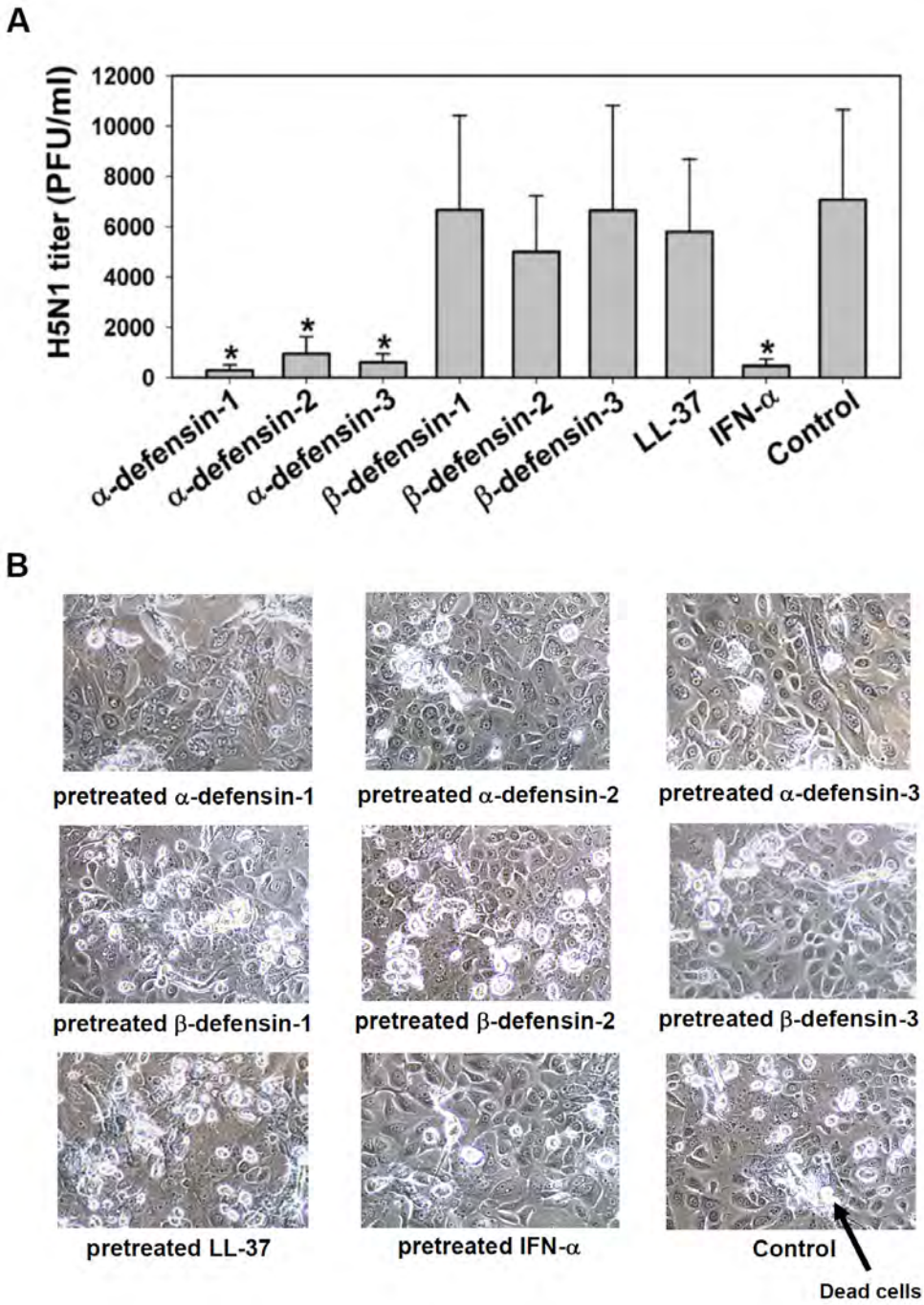

Figure 9. Protection against avian influenza H5N1infection by MxA protein in α-defensin -pretreated HGECs. (A) HGECs pretreated for 24 h with either α-defensin-1; α-defensin-2; α-defensin-3; β-defensin-1 ; β-defensin-2; β-defensin-3; LL37; IFN-α; or not pretreated were co-cultured with H5N1 virus at MOI 1. Viral titers in culture supernatant were determined at 48 h post-infection in HGECs as described in Materials and methods. Data are mean PFU/ml ± SEM of four separate experiments. One sample per experiment and each sample derived from

different individual donors (*, $p < 0.05$, compared with control). (B) As in (A) but treated cells were analysed by light microscopy. Dead cells were rounded and differentiated from the polygonalshaped healthy cells. Data are representative of four separate experiments. One sample per experiment and each sample derived from different individual donors.

To confirm the antiviral activity of MxA against H5N1, we transfected HGECs with MxA-targeted siRNA, treated the cells with α-defensin-1 overnight, and then infected them with H5N1 virus. MxA-targeted siRNA greatly reduced levels of MxA mRNA expression, by 95%, (Fig. 10A) and effectively abolished inhibition of viral replication, by 93%, in H5N1-infected HGECs (Fig. 10B). These findings were supported by microscopically observed cytopathic effects (Fig. 10C).

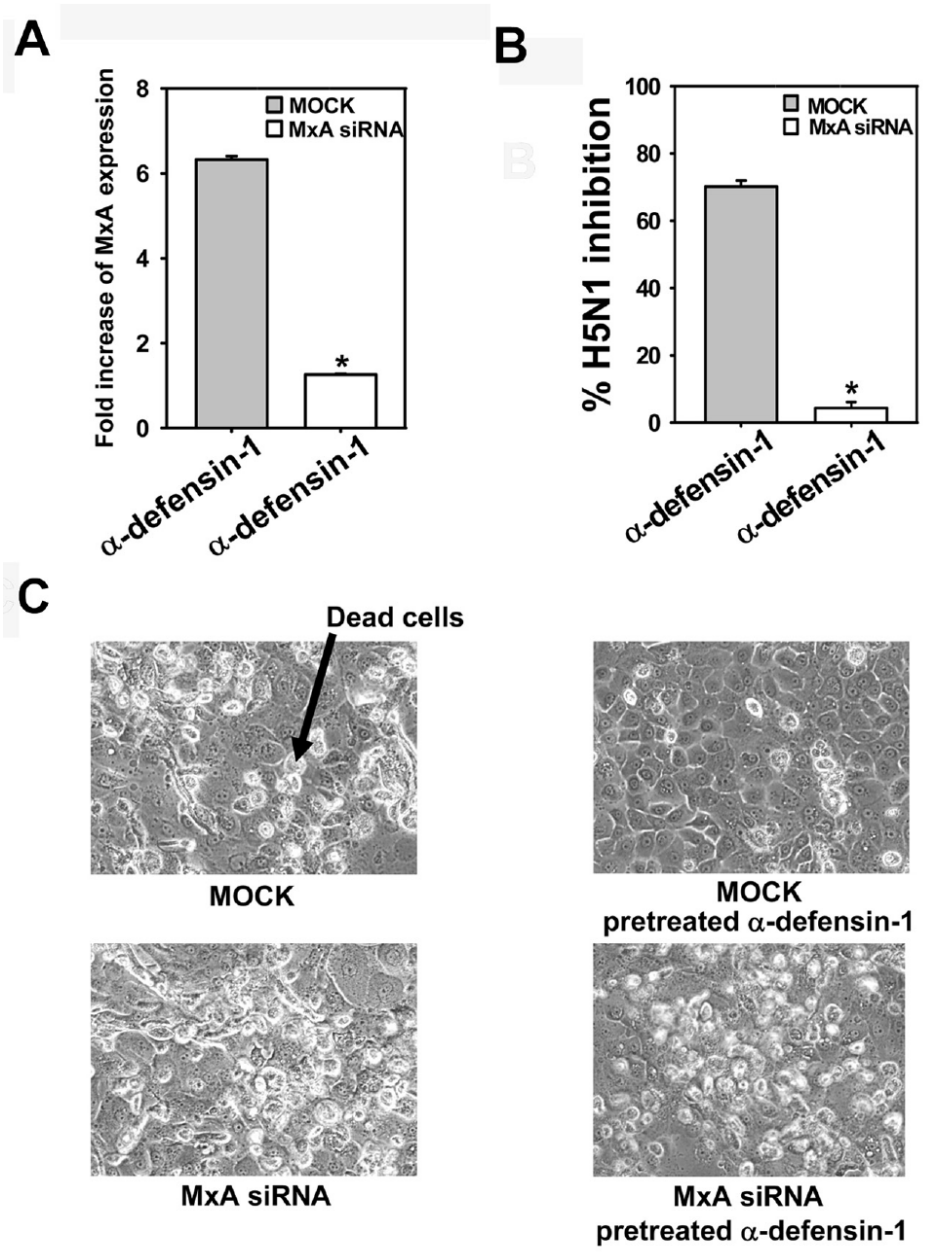

Figure 10. Protection against avian influenza H5N1infection by MxA siRNA in α-defensin -pretreated HGECs. (A) HGECs were transfected with MxA siRNA or MOCK control and then treated with α-defensin-1. Real-time RT-PCR was used to analyze MxA mRNA expression in the cells. Data are demonstrated as the mean fold increase ± SEM of MxA mRNA expression in α-defensin-1-treated HGECs that had been transfected with MxA siRNA or MOCK control, compared with medium control after normalization with the GAPDH of four separate experiments . One sample per experiment and each sample derived from different individual donors (*, $p < 0.05$, compared with MOCK control). (B) As in (A) but the transfected HGECs, which were treated with α-defensin-1, were then co-

cultured with H5N1 virus at MOI 1 and inhibition of viral replication was subsequently measured. Data are mean percentage of H5N1 inhibition ± SEM of four separate experiments. One sample per experiment and each sample derived from different individual donors (*, $p < 0.05$, compared with MOCK control). (C) As in (B) but treated cells were analysed by light microscopy. Dead cells were rounded and differentiated from the polygonalshaped healthy cells. Data are representative of four separate experiments. One sample per experiment and each sample derived from different individual donors.

PMN-derived-α-defensins induce MxA expression in HGECs

α-defensins are known as major proteins secreted by PMNs (Ganz 2003). In the physiological condition of healthy gingiva, PMNs and their products are present in the tissue and the crevicular fluid in the gingival sulcus (Lundy et al., 2005; Dale et al., 2001). *In vitro* culture of PMNs (5×10^6 cells/ml) for 6 h led to secretion of α-defensins in supernatants (which ranged from 90,479 to 98,714 pg/ml). To investigate the role of the PMN-derived α-defensins in MxA expression, we cultured HGECs with 6 h PMN supernatants. Under this condition, expression of MxA at both mRNA and protein levels in HGEC was observed after 6 h and 24 h treatment, respectively (Fig 11A and 11B). The MxA-inducing activity was diminished when neutralizing antibody against α-defensins was added to the culture, whereas neutralizing antibodies against type I IFN (IFN-α and IFN-β) had no effect (Fig. 11B). These data suggest that PMN-derived α-defensins were responsible for the observed MxA expression.

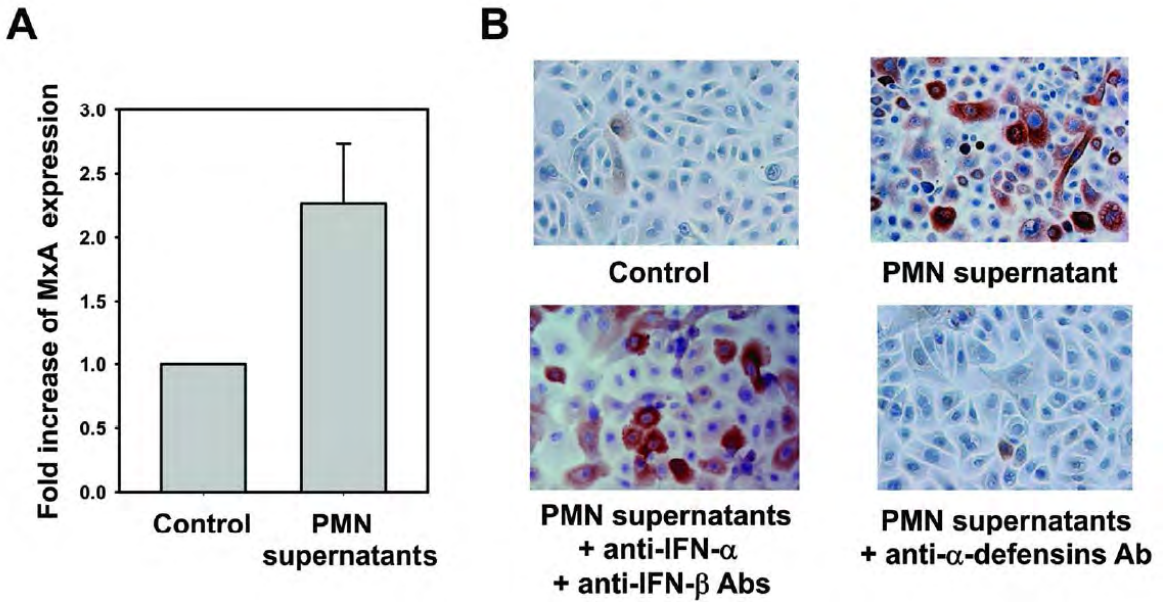

Figure 11. PMN-derived α-defensins induced MxA expression in HGECs. (A) Real-time RT-PCR was used to analyze mRNA expression of MxA in HGEC cultures treated with PMN culture supernatants. Culture medium was

used as negative control. Data are demonstrated as the mean fold increase ± SEM of MxA mRNA expression after normalization with the GAPDH of four separate experiments. One sample per experiment and each sample derived from different individual donors. Control is set to 1. (B) Immunohistochemical staining with anti-MxA antibody was used to detect MxA in HGEC cultures treated with either: PMN culture supernatants; PMN culture supernatants plus neutralizing antibodies against type I IFN (IFN-α and IFN-β); or PMN culture supernatants plus neutralizing antibodies against α-defensins. Culture medium was used as negative control. Data are representative of four separate experiments. One sample per experiment and each sample derived from different individual donors.

MxA and α-defensin protein expression in healthy gingival sulcus

The immunostaining results to detect epithelial MxA were obtained using the oral, but not the sulcus, side of periodontal tissue (Fig. 1) because the epithelium at the sulcus side, especially for the junctional epithelium, is generally lost or torn during the surgical procedure. Fig. 12A depicts anatomic landmarks of the gingival sulcus. In this study, we were able to obtain two specimens of gingival sulcus area from healthy periodontal tissue. We then investigated localization of MxA protein in the healthy sulcus and also in relation to α-defensin. Fig. 12C shows that MxA protein was consistently expressed throughout epithelial cells of periodontal tissues. MxA staining was especially intense in the junctional epithelium (Fig. 12C). α-defensins were identified in small round cells with PMN morphology, most of which were found in the connective tissue layer (Fig. 12E). Migratory PMNs in junctional epithelium were also observed and highlighted in Fig. 13D. Thus, epithelial MxA protein was in close association with α-defensin-positive cells.

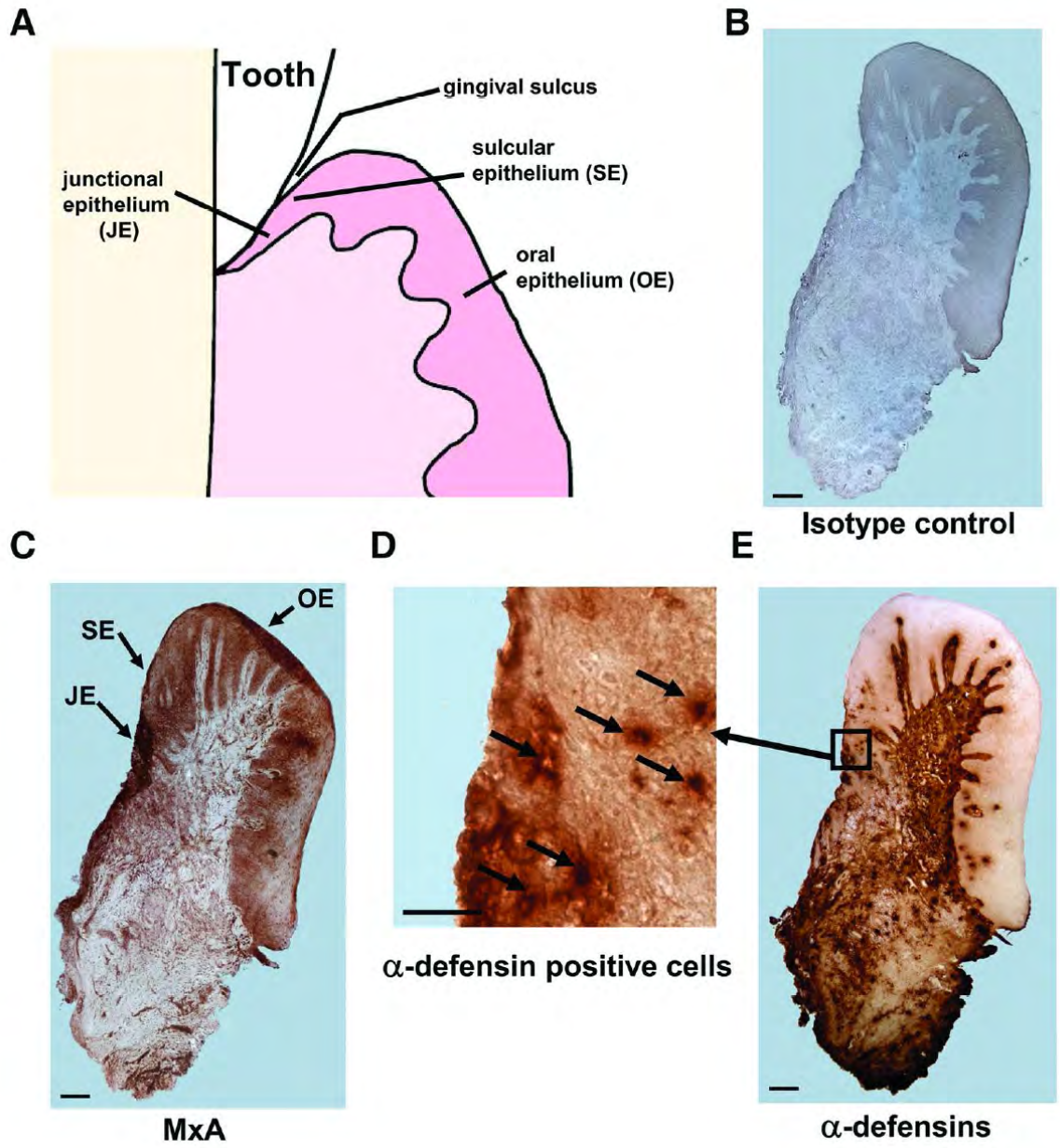

Figure 12. MxA and α-defensin protein expression in healthy gingival sulcus. (A) Diagram showing anatomic landmarks of the gingival sulcus area. Healthy periodontal tissue including the gingival sulcus area was stained with (B) isotype control, (C) anti-MxA antibody, and (D, E) anti-α-defensin antibody by immunoperoxidase technique and counterstained with haematoxylin. Data are representative of two different individual tissue specimens. Scale bars represent 200 μM in B, C, and E, and 50 μM in D.

References

Brand, S., Beigel, F., Olszak, T., Zitzmann, K., Eichhorst, S. T., Otte, J. M., Diebold, J. *et al*., (2005) IL-28A and IL-29 mediate antiproliferative and antiviral signals in intestinal epithelial cells and murine CMV infection increases colonic IL-28A expression. *Am J Physiol Gastrointest Liver Physiol* 289: G960-968.

Dale, B. A., Kimball, J. R., Krisanaprakornkit, S., Roberts, F., Robinovitch, M., O'Neal, R., Valore, E. V. *et al*., (2001) Localized antimicrobial peptide expression in human gingiva. *J Periodontal Res* 36: 285-294.

Diamond, G., Beckloff, N. and Ryan, L. K. (2008) Host defense peptides in the oral cavity and the lung: similarities and differences. *J Dent Res* 2008. 87: 915-927.

Falco, A., Mas, V., Tafalla, C., Perez, L., Coll, J. M. and Estepa, A., (2007) Dual antiviral activity of human alpha-defensin-1 against viral haemorrhagic septicaemia rhabdovirus (VHSV): inactivation of virus particles and induction of a type I interferon-related response. *Antiviral Res* 76: 111-123.

Ganz, T. (2003) Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol* 3: 710-720.

Haller, O., Frese, M. and Kochs, G. (1998) Mx proteins: mediators of innate resistance to RNA viruses. *Rev Sci Tech* 17: 220-230.

Holzinger, D., Jorns, C., Stertz, S., Boisson-Dupuis, S., Thimme, R., Weidmann, M., Casanova, J. L. *et al*., (2007) Induction of MxA gene expression by influenza A virus requires type I or type III interferon signaling. *J Virol* 81: 7776-7785.

Jana, N. K., Gray, L. R. and Shugars, D. C. (2005) Human immunodeficiency virus type 1 stimulates the expression and production of secretory leukocyte protease inhibitor (SLPI) in oral epithelial cells: a role for SLPI in innate mucosal immunity. *J Virol* 79: 6432-6440.

Lundy, F. T., Orr, D. F., Shaw, C., Lamey, P. J. and Linden, G. J. (2005) Detection of individual human neutrophil alpha-defensins (human neutrophil peptides 1, 2 and 3) in unfractionated gingival crevicular fluid--a MALDI-MS approach. *Mol Immunol* 42: 575-579.

Netherton, C. L., Simpson, J., Haller, O., Wileman, T. E., Takamatsu, H. H., Monaghan, P. and Taylor, G. (2009) Inhibition of a large double-stranded DNA virus by MxA protein. *J Virol* 83: 2310-2320.

Pavlovic, J., Haller, O. and Staeheli, P. (1992). Human and mouse Mx proteins inhibit different steps of the influenza virus multiplication cycle. *J Virol* 66: 2564-2569.

Discussion

To the best of our knowledge, the present study is the first to identify in humans the ability of α-defensins, endogenous antimicrobial peptides from PMNs, to induce the expression of epithelial MxA, a potent antiviral protein against both RNA and DNA viruses. This innate antiviral immune mechanism could play an important role in maintaining healthy periodontal tissues. α-defensin-induced MxA is an additional pathway to the well-recognized type I IFN induction (von Wussow et al., 1990; Ronni et al., 1993). This function seems to be unique to α-defensins, because other antimicrobial peptides in healthy periodontal tissue (β-defensins and LL37) induced only negligible MxA expression. It should be noted that α-defensins are known to up-regulate co-stimulatory molecule and CD91 expression on antigen presenting dendritic cells (Presicce et al., 2009).

There is little available information regarding innate antiviral immunity in the oral cavity. The human mouth harbors millions of microbes; however, we rarely develop serious infections (Paster et al., 2001). Our previous research demonstrated TLRs and RLRs, key microbial sensors, in cells of periodontal tissues, which are critical for innate immune activation and local defense (Mahanonda and Pichyangkul, 2007; Mahanonda et al., 2007; Mahanonda et al., 2009. In the present study we observed expression of MxA, PKR, OAS, and SLPI in healthy periodontal tissues, thus highlighting the role of innate antiviral immunity in periodontal tissue.

MxA proteins are key mediators of innate antiviral resistance induced in cells by type I (α/β) and type III (λ) IFNs (Holzinger et al., 2007). The human *MxA* gene belongs to the class of IFN-stimulated genes (ISGs) and it is used as a surrogate marker for type I IFN activity in various experimental and clinical settings. Santoro et al., (2005) used MxA to identify type I IFN in oral lichen planus. They found large numbers of MxA positive cells in the lesion, therefore a role of type I IFN in the pathology of oral lichen planus was postulated.

We are unaware of any previous study of MxA in periodontal disease. Our consistent finding of positive immunostaining of MxA protein in epithelium of healthy periodontal tissues (n = 9) was somewhat unexpected, since real-time PCR detected only negligible expression of type I IFN or type III IFN in healthy tissue specimens. Interestingly the level of MxA proteins in the epithelial layer was significantly higher in healthy periodontal tissues than in periodontitis (Table 1). While searching for candidate MxA inducers, we treated primary HGECs with a variety of antimicrobial molecules, which are constitutively expressed in gingival epithelium. We clearly observed MxA protein expression after treatment with α-defensins-1, 2, or 3, but not with the other antimicrobial peptides β-defensins-1, 2, 3, or LL37.

At present, it is not clear how α-defensins induce MxA expression. Our data strongly suggest that induction of MxA expression by α-defensin-1 is not dependent on type I IFN as neutralizing antibodies against type I IFN had no effect on the MxA expression. The mechanism of α-defensin-1-induced MxA expression is

different from IFN-α-induced MxA expression since we could not detect the enhanced expression of other ISGs including STAT1, STAT2, IRF3, IRF7, and IRF9. Furthermore, STAT1 activation was also not detected in α-defensin-1-treated HGECs. This observation is in line with previous study which showed that α-defensin-1 did not induce STAT1 activation in HeLa-CD4 cells (Chang et al., 2003). The α-defensin-1-induced MxA expression was not specific to HGECs since this effect was also observed in normal human bronchial epithelial cells and primary human microvascular endothelial cells. These findings are supported by recent observations showing that human α-defensin-1 induced homologue MxA in fish cell line (Falco et al., 2007. Our results may also explain the previous observation which demonstrated that MxA can be induced in LPS-stimulated PMNs independent of type I IFN (Malcolm and Worthen, 2003). It is possible that LPS stimulated PMNs to release α-defensins, resulting in MxA expression.

MxA is a protein with broad antiviral activity; it blocks viral replication at an early stage (Haller, Staeheli and Kochs, 2009). We demonstrated that MxA expressed in α-defensin-treated HGECs inhibited avian influenza H5N1 viral replication. After silencing the *MxA* gene, HGECs treated with α-defensin-1 robustly down-regulated MxA function, allowing viral replication and cell death to occur. It is tempting to speculate that MxA expression in periodontal tissue may have a role in antiviral defense during the consumption of H5N1-infected poultry meat; however, further research is required. It should be noted that α-defensins are known to directly inactivate viruses and inhibit their entry (Daher, Selsted and Lehrer, 1986). Our results provide additional antiviral pathway by which α-defensins modulate host cells to express MxA protein and inhibit viral replication.

PMNs are a major source of α-defensins. Our in vitro data demonstrated that when neutralizing antibody against α-defensins was added to the PMN supernatant-treated HGECs culture, the MxA-inducing activity was diminished. Therefore, α-defensins released from PMNs are likely to be responsible for the observed MxA expression in periodontal tissue. The intense MxA staining observed in the gingival sulcus area may be related to the pathway of a constant migration of PMNs from subepithelial connective tissue vessels through junctional epithelium and into this area (Bosshardt and Lang, 2005). This dynamic sequence suggests a crosstalk between resident non-immune cells, the epithelium, and professional phagocytic cells, PMNs, all of which are essential for local innate immune activation.

It is interesting to note that MxA expression was lower in diseased periodontal tissue which commonly has more infiltrated PMNs as compared with healthy periodontal tissue. One could speculate that down-regulation of the observed MxA expression in periodontitis could result from suppressor proteins produced during intense inflammatory reaction. The increased expression of suppressors of cytokine signaling (SOCS) proteins in periodontitis was recently reported (Garlet et al., 2006. Both SOCS-1 and SOCS-3 are able to inhibit MxA expression (Vlotides et al., 2004).

In conclusion, this study demonstrates that α-defensins, antimicrobial peptides constitutively expressed in healthy periodontal tissue, induces expression of a classical antiviral protein, MxA, in gingival epithelium. Strong MxA activity at the strategic gingival sulcus, in close proximity to microbial plaque, may serve as one of the important innate tools in maintaining periodontal homeostasis. We believe that our findings warrant further research into the physiological role of α-defensin-induced MxA in the antiviral response of the periodontal tissue.

References

Bosshardt, D. D. and Lang, N. P. (2005) The junctional epithelium: from health to disease. *J Dent Res* 84: 9-20.

Chang, T. L., Francois, F., Mosoian, A. and Klotman, M. E., (2003) CAF-mediated human immunodeficiency virus (HIV) type 1 transcriptional inhibition is distinct from alpha-defensin-1 HIV inhibition. *J Virol* 77: 6777-6784.

Daher, K. A., Selsted, M. E. and Lehrer, R. I. (1986) Direct inactivation of viruses by human granulocyte defensins. *J Virol* 60: 1068-1074.

Falco, A., Mas, V., Tafalla, C., Perez, L., Coll, J. M. and Estepa, A., (2007) Dual antiviral activity of human alpha-defensin-1 against viral haemorrhagic septicaemia rhabdovirus (VHSV): inactivation of virus particles and induction of a type I interferon-related response. *Antiviral Res* 76: 111-123.

Garlet, G. P., Cardoso, C. R., Campanelli, A. P., Martins, W., Jr. and Silva, J. S. (2006) Expression of suppressors of cytokine signaling in diseased periodontal tissues: a stop signal for disease progression? *J Periodontal Res* 41: 580-584.

Haller, O., Staeheli, P. and Kochs, G. (2009) Protective role of interferon-induced Mx GTPases against influenza viruses. *Rev Sci Tech* 28: 219-231.

Holzinger, D., Jorns, C., Stertz, S., Boisson-Dupuis, S., Thimme, R., Weidmann, M., Casanova, J. L. *et al*., (2007) Induction of MxA gene expression by influenza A virus requires type I or type III interferon signaling. *J Virol* 81: 7776-7785.

Mahanonda, R. and Pichyangkul, S. (2007) Toll-like receptors and their role in periodontal health and disease. *Periodontol 2000* 43: 41-55.

Mahanonda, R., Sa-Ard-Iam, N., Montreekachon, P., Pimkhaokham, A., Yongvanichit, K., Fukuda, M. M. and Pichyangkul, S. (2007) IL-8 and IDO expression by human gingival fibroblasts via TLRs. *J Immunol* 178: 1151-1157.

Mahanonda, R., Sa-Ard-Iam, N., Eksomtramate, M., Rerkyen, P., Phairat, B., Schaecher, K. E., Fukuda, M. M. and Pichyangkul, S. (2009) Cigarette smoke extract modulates human beta-defensin-2 and interleukin-8 expression in human gingival epithelial cells. *J Periodontal Res* 2009. 44: 557-564.

Malcolm, K. C. and Worthen, G. S. (2003) Lipopolysaccharide stimulates p38-dependent induction of antiviral genes in neutrophils independently of paracrine factors. *J Biol Chem* 278: 15693-15701.

Paster, B. J., Boches, S. K., Galvin, J. L., Ericson, R. E., Lau, C. N., Levanos, V. A., Sahasrabudhe, A. and Dewhirst, F. E. (2001) Bacterial diversity in human subgingival plaque. *J Bacteriol* 183: 3770-3783.

Presicce, P., Giannelli, S., Taddeo, A., Villa, M. L. and Della Bella. S. (2009) Human defensins activate monocyte-derived dendritic cells, promote the production of proinflammatory cytokines, and up-regulate the surface expression of CD91. *J Leukoc Biol* 86(4): 941-948.

Ronni, T., Melen, K., Malygin, A. and Julkunen, I. (1993) Control of IFN-inducible MxA gene expression in human cells. *J Immunol* 150: 1715-1726.

Santoro, A., Majorana, A., Roversi, L., Gentili, F., Marrelli, S., Vermi, W., Bardellini, E. *et al.*, (2005) Recruitment of dendritic cells in oral lichen planus. *J Pathol* 205: 426-434.

Vlotides, G., Sorensen, A. S., Kopp, F., Zitzmann, K., Cengic, N., Brand, S., Zachoval, R. and Auernhammer, C. J. (2004) SOCS-1 and SOCS-3 inhibit IFN-alpha-induced expression of the antiviral proteins 2,5-OAS and MxA. *Biochem Biophys Res Commun* 320: 1007-1014.

von Wussow, P., Jakschies, D., Hochkeppel, H. K., Fibich, C., Penner, L. and Deicher, H. (1990) The human intracellular Mx-homologous protein is specifically induced by type I interferons. *Eur J Immunol* 20: 2015-2019.

Appendices

Reprint form Periodontology 2000

Periodontology 2000, Vol. 56, 2011, 143–153
Printed in Singapore. All rights reserved

Innate antiviral immunity of periodontal tissue

Rangsini Mahanonda, Noppadol Sa-Ard-Iam, Pimprapa Rerkyen, Chantrakorn Champaiboon, Narisara Vanavit & Sathit Pichyangkul

The human mouth harbors millions of highly diverse microbial organisms. These include approximately 700 species of bacteria, as well as viruses, fungi and protozoa (20, 79). These microbes outnumber the cells of the mucosal lining. They continually live and grow in an oral ecosystem that provides moisture, warmth and nutrients. Even though the oral mucosa is constantly exposed to a variety of microbes, most individuals maintain homeostasis, suggesting an effective innate immune system in the oral cavity.

The etiological importance of bacteria in periodontal disease has been understood for decades. Bacterial plaque biofilms continually form on the tooth surfaces adjacent to gingiva. Gram-positive bacteria, aerobes and facultative anaerobes, including *Streptococcus*, *Veillonella* and *Actinomyces* species, are a major component of dental plaque and in the gingival sulcus of healthy and inflamed gingiva. Bacterial numbers are lower at healthy sites than inflamed ones. In contrast, most microbial plaques associated with periodontitis lesions comprise gram-negative anaerobes and facultative anaerobes such as *Porphyromonas gingivalis*, *Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans* and *Treponema denticola* (34, 42, 70, 102, 104).

Several studies have also documented a role for viruses in the development and progression of periodontitis and other oral infections. In particular, herpesviruses, a group of DNA viruses, have emerged as putative periodontal pathogens (103). Herpesviruses are commonly found in the oral cavity, and most individuals become infected with herpesviruses in childhood. DNA from herpesviruses such as herpes simplex virus type 1, herpes simplex virus type 2, human cytomegalovirus and Epstein–Barr virus (59) has been detected in subgingival plaque (14, 96, 97), gingival crevicular fluid (14, 77), gingival tissue (13, 24) and infiltrated immune cells from periodontitis sites (16). Viral DNA can also be found at healthy sites, but generally at a lower frequency than at periodontitis sites (13, 16, 77). Herpesvirus infection varies considerably with regard to their clinical outcomes, which range from sub-clinical to potentially lethal infections such as pneumonia, encephalitis and cancer, including carcinoma and sarcoma (3, 72, 78, 88, 117). Most human adults are carriers of human cytomegalovirus and Epstein–Barr virus. Both of these have been frequently associated with periodontal diseases such as chronic periodontitis (96), aggressive periodontitis (52, 118), apical periodontitis lesions (91) and periodontal abscess (97). mRNA transcription of human cytomegalovirus has been demonstrated in deep periodontal pockets of chronic periodontitis and in localized juvenile periodontitis, but not in shallow periodontal sites, suggesting activation of human cytomegalovirus in periodontitis (15, 109).

Other viruses such as human papillomavirus (59, 77), human immunodeficiency virus (HIV) (14, 77) and hepatitis C virus (67) have also been identified at periodontal disease sites. However, their role in periodontal disease is not yet understood, and requires further investigation.

Innate antiviral immune response in the oral cavity

Innate immunity serves as the first line of defense against invading microorganisms. It consists of non-cellular and cellular components. The innate antibacterial immune response in the oral cavity has been the focus of considerable recent research (98, 107, 122). However, to date, there is relatively little information available regarding oral innate antiviral immunity. Non-cellular and cellular components of

this immunity are discussed below. The roles of secretory antiviral proteins in saliva, and of the oral mucosal epithelium as a viral sensor and terminator, are highlighted.

Non-cellular components in saliva

Saliva plays a key role in reducing the accessibility of microbe-susceptible cells in the oral cavity. In addition to lubricating the oral cavity, saliva contains a variety of non-cellular innate mediators with antimicrobial properties. Examples of these saliva-soluble factors are mucins (76), amylase (76), proline-rich proteins (76), histatins (116), statherin (116), salivary gp-340 (or salivary agglutinin) (76) and lysozyme (69). Saliva also contains a variety of other potential host defense peptides and proteins, including human α-defensins (commonly known as human neutrophil peptides) (32), human β-defensins (66), cathelicidin (LL37) (73), thrombospondins (18), lactoferrin (69) and secretory leukocyte protease inhibitor (69). Most studies have focused on the anti-bacterial activities of these saliva-soluble substances. However, many of them have been reported to have antiviral properties as well. The known innate antiviral components of saliva are listed in Table 1, which also includes the types of affected viruses and the mechanisms of viral inhibition.

Cellular components

The oral mucosa forms an effective physical barrier. The thick, multi-layered epithelia appear to tolerate the attachment of microbes on the dead superficial epithelial layers, which are continuously sloughed off (101). Keratin, which coats most of the oral mucosa, provides a barrier against viral infection (26). Additionally, oral epithelial cells express pattern recognition receptors that detect early viral invasion, leading to the production of mediators that terminate viral infection.

Upon entry into a host cell, a virus is recognized by the innate immune system. Viral nucleic acids serve as viral pathogen-associated molecular patterns, which are recognized by at least two distinct families of pattern recognition receptors, i.e. Toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) as shown in Fig. 1. Within the TLR family, TLR3, TLR7, TLR8 and TLR9 represent a sub-family that is localized to intracellular compartments, such as the endosome and endoplasmic reticulum, and that recognizes nucleic acid species (49, 55, 74, 80). Other TLRs (TLR1, TLR2, TLR4, TLR5 and TLR6) are localized on the cell surface, and mainly recognize bacterial cell wall components or viral particles. TLR3 recognizes double-stranded viral RNA. TLR7 and TLR8 identify single-stranded viral RNA (TLR8 is the TLR that is phylogenetically most similar to TLR7). TLR9 detects viral DNA. Upon recognition of these nucleic acid species, TLRs recruit specific intracellular adaptor proteins to initiate signaling pathways. TLR3 relies on the adaptor Toll/interleukin-1R-containing adaptor inducing interferon-β. This pathway leads to activation of transcription factor interferon regulatory factor 3 (IRF3) resulting in production of type I interferon, and activation of nuclear factor κB (NF-κB), resulting in induction of inflammatory cytokines such as interleukin-6, interleukin-12 and tumor necrosis factor-α. Unlike TLR3, TLR7/TLR8 and TLR9 use myeloid differentiation primary response gene 88 (MyD88) as an adaptor. This MyD88-dependent pathway leads to activation of transcription factor IRF7 to induce production of type I interferon, and activation of NF-κB to induce inflammatory cytokines (49). The most important antiviral mediator, type I interferon, plays an essential role in the elimination of viruses. It enhances the transcription of many interferon-inducible genes that influence protein synthesis, viral growth arrest and apoptosis. Type I interferon also enhances dendritic cell maturation, antibody production and differentiation of virus-specific cytotoxic T lymphocytes, resulting in effective adaptive immunity against viral infection (43, 108).

TLRs that are localized to endosomes are unable to sense viruses once they have entered the cytosol. RLRs, which are cytoplasmic RNA helicases, utilize TLR-independent mechanisms to sense double-stranded and single-stranded viral RNA (50). RLRs include the products of RIG-I, melanoma differentiation associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) (90, 119, 120). During RIG-I and MDA5 signaling, interferon-β promoter stimulatory-1 functions as an adaptor that mediates activation of IRF3 and IRF7 to induce type I interferon, and activation of NF-κB to induce inflammatory cytokines (50) (Fig. 1). Other pattern recognition receptors that sense viral pathogen-associated molecular patterns have also been identified recently. These include a cytosolic DNA sensor termed DNA-dependent activator of IRF (106) and NACHT-LRR-PYD-domain containing protein 3 (NALP 3) (48) (Fig. 1). Further investigation is required to explain how pattern recognition receptors detect nucleic acid and induce antiviral innate immune responses.

Table 1. Saliva-associated antiviral molecules

Saliva components	Viral inhibition	Mechanisms and references
HBD-1	HSV-2	No inhibition (39)
	HIV-1	No inhibition (85)
HBD-2	HSV-2	No inhibition (39)
	HIV-1	Interferes with viral entry, modulates host cells, inhibits viral replication (85)
HBD-3	HSV-2	Interferes with viral entry (39)
	HIV-1	Interferes with viral entry, modulates host cells, inhibits viral replication (85)
HNP-1	HSV-1	Interferes with viral entry (19), modulates host cells, inhibits viral replication (39)
	HSV-2	Interferes with viral entry (19, 39, 47), modulates host cells, inhibits viral replication (39, 47)
	HIV-1	Modulates host cells, inhibits viral replication (11, 58, 123), interferes with viral entry (12, 58)
	HMCV	Interferes with viral entry (19)
	Influenza A	Interferes with viral entry (19, 116)
	Papillomavirus	Modulates host cells, inhibits viral replication (10)
HNP-2	HSV-1	Modulates host cells, inhibits viral replication (39), interferes with viral entry (19)
	HSV-2	Interferes with viral entry, modulates host cells, inhibits viral replication (39, 47)
	HIV-1	Modulates host cells, inhibits viral replication (123)
	Influenza A	Interferes with viral entry (116)
	Papillomavirus	Modulates host cells, inhibits viral replication (10)
HNP-3	HSV-1	Interferes with viral entry (19), modulates host cells, inhibits viral replication (39)
	HSV-2	Interferes with viral entry, modulates host cells, inhibits viral replication (39, 47)
	HIV-1	Modulates host cells, inhibits viral replication (123)
	Papillomavirus	Modulates host cells, inhibits viral replication (10)
LL37	HIV	Modulates host cells, inhibits viral replication (7)
	Papillomavirus	Modulates host cells, inhibits viral replication (10)
Agglutinin gp340	Influenza A	Interferes with viral entry (116)
Histatins	Influenza A	Interferes with viral entry (116)

Table 1. (*Continued*)

Saliva components	Viral inhibition	Mechanisms and references
Thrombospondin	HIV-1	Interferes with viral entry (18)
	Influenza A	Interferes with viral entry (116)
Basic proline-rich protein	HIV-1	Interferes with viral entry (87)
Acidic proline-rich protein	Influenza A	Interferes with viral entry (116)
Lactoferrin	HSV-1	Interferes with viral entry (65), inhibits viral replication (113)
	HSV-2	Interferes with viral entry (63)
	HIV-1	Interferes with viral entry (37, 82)
	CMV	Interferes with viral entry (1, 38)
	Papillomavirus	Interferes with viral entry (23), modulates host cells, inhibits viral replication (10)
	Hepatitis C virus	Interferes with viral entry (44, 75)
	Poliovirus 1	Interferes with viral entry (64)
	RSV	Interferes with viral entry (94)
	Influenza A	Interferes with viral entry (116)
SLPI	HSV-2	Interferes with viral entry, modulates host cells, inhibits viral replication (47)
	HIV-1	Interferes with viral entry (84, 100, 115)
	Influenza A	Interferes with viral entry (6)
	Sendai virus	Interferes with viral entry (6)
Mucin	Hantavirus	Suggested interference with viral entry (36)
Mucin 5B	Influenza A	Interferes with viral entry (116)

CMV, cytomegalovirus; HBD, human β-defensin; HIV, human immunodeficiency virus; HSV, herpes simplex virus; HNP, human neutrophil peptide; LL37, cathelicidin; RSV, respiratory syncytial virus; SLPI, secretory leukocyte protease inhibitor.

TLR recognition of viruses associated with oral disease has been reported (Fig. 1). Herpes simplex virus type 1, herpes simplex virus type 2 (41, 51, 56), and gammaherpesvirus (MHV-68- Epstein–Barr virus, similar to human Epstein–Barr virus) (33) are recognized by TLR9. Cytomgalovirus is recognized by TLR3 and TLR9 (105), whereas HIV is recognized by TLR7 and TLR8 (4, 40). Glycoproteins in the viral envelope can be recognized as pathogen-associated molecular patterns by cell-surface TLRs. For example, envelope glycoproteins of herpes simplex virus type 1, human cytomegalovirus and Epstein–Barr virus are recognized by TLR2 (2, 8, 31, 54). Recently, Sato et al. (95) proposed a dual action of TLR2 and TLR9 for recognition of herpes simplex virus by dendritic cells. Activation of dual or multiple TLRs by a single pathogen may be advantageous to the host, as it could allow the immune system to more precisely identify an invading pathogen and then mount an appropriate response.

As reviewed by Mahanonda & Pichyangkul (60) and observed by others (61, 62), cells of periodontal tissues (gingival epithelial cells, gingival fibroblasts, periodontal ligament fibroblasts, cementoblasts and osteoblasts) express a variety of TLRs. Primary human gingival epithelial cells derived from healthy periodontal tissues express mRNA for the TLRs 1, 2, 3, 4, 5, 6 and 9, but not TLRs 7 or 8 (61). After triggering by polyinosinic/polycytidylic acid (a synthetic analog of double-stranded RNA that triggers TLR3), human gingival epithelial cells express and secrete human β-defensin-2 and interleukin-8, suggesting the presence of a functional TLR3 (61). Polyinosinic/polycytidylic acid also induces the antiviral response of human gingival epithelial cells via interferon-β production (unpublished data). On the other hand, TLR7 and TLR8 ligands are not able to induce

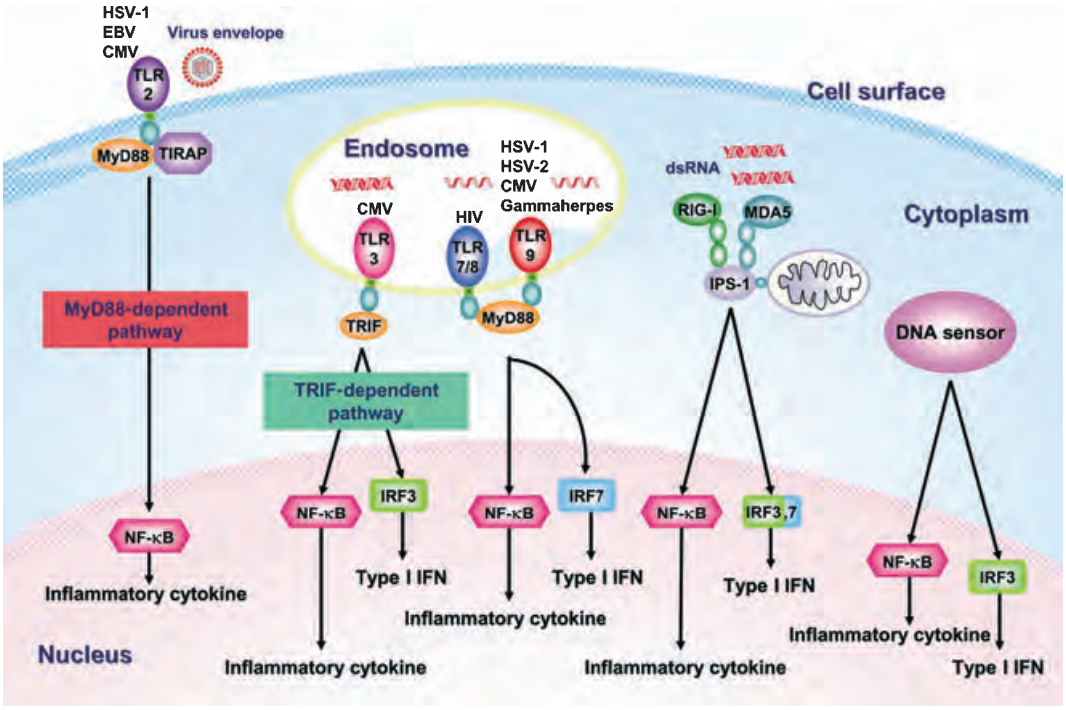

Fig. 1. Signaling pathways of viral sensing receptors (TLRs, RLRs and DNA sensors) (simplified diagram) and viral pathogen-associated molecular patterns associated with oral diseases. CMV, cytomegalovirus; EBV, Epstein–Barr virus; HIV, human immunodeficiency virus; HSV, herpes simplex virus; IFN, interferon; IPS-1, IFN-β promoter stimulatory-1; IRF, IFN regulatory factor; MDA5, melanoma differentiation associated gene 5; MyD88, myeloid differentiation primary response gene 88; NF-κB, nuclear factor κB; RIG-I, retinoic acid-indcible gene I; TIR, Toll/interleukin-1 receptor; TIRAP, TIR domain-containing adaptor protein; TLRs, Toll-like receptors; TRIF, TIR-containing adaptor inducing IFN-β.

expression of human β-defensin-2 or interleukin-8, confirming the absence of TLR7 and TLR8 expression in human gingival epithelial cells. Interestingly, human gingival epithelial cells clearly express TLR9 but do not respond to the synthetic TLR9 ligand CpG oligodeoxynucleotide in *in vitro* cell cultures (61). The reason for a non-functional TLR9 in human gingival epithelial cells remains unclear and requires further investigation.

In contrast, other investigators have demonstrated expression of TLR7 and TLR8 in both healthy and periodontitis tissues using immunohistochemical staining (5). We are currently attempting to replicate this finding. If the finding is confirmed, it would further support a role of the antiviral innate immune response in gingival epithelial cells.

In addition to the presence of viral-sensing TLRs, a few studies have recently identified RLRs in periodontal cells. RIG-I expression was detected in human gingival fibroblasts (53, 92), and this could be up-regulated by lipopolysaccharide and double-stranded RNA (53). Based on our previously unpublished data shown in Fig. 2, both human gingival epithelial cells and human gingival fibroblasts derived from healthy gingiva express mRNA for RIG-I and MDA5.

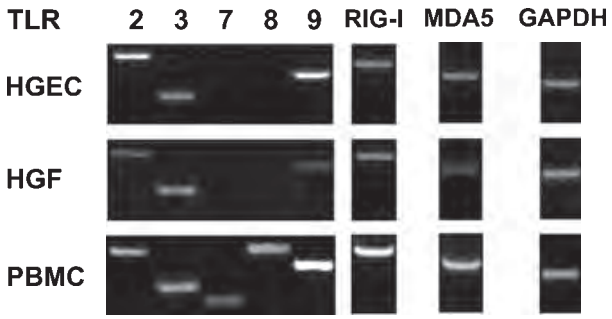

Fig. 2. mRNA expression of TLRs and RLRs for viral pathogen-associated molecular patterns in healthy periodontal cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HGEC, human gingival epithelial cells; HGF, human gingival fibroblasts; MDA5, melanoma differentiation associated gene 5; PBMC, peripheral blood mononuclear cells; RIG-I, retinoic acid-inducible gene I; TLR, Toll-like receptor.

The gingival sulcus: a strategic area in periodontal innate immunity

The gingival sulcus is a strategically important location in which periodontal tissue is constantly challenged by microbial biofilms and their products. In healthy gingiva, the gingival sulcus is a shallow,

v-shaped crevice (30). It is bounded by the tooth surface on one side and the sulcular epithelium on the other. At the bottom lies the junctional epithelium. The integrity of the junctional epithelium and the sulcular epithelium is crucial for maintaining periodontal health. Conversion of the junctional epithelium to pocket epithelium is widely regarded as a hallmark for loss of periodontal attachment and the development of periodontitis (9).

Unlike the conventional stratified squamous keratinizing epithelium that lines the outer gingival aspect (oral epithelium), both the sulcular and junctional epithelium are non-keratinizing and thin (25), and are apparently more susceptible to infection. Hence, efficient innate mechanisms against microbial invasion are essential at such sensitive sites. Recent research has shown that the junctional epithelium may play a much more active role in innate immunity than previously assumed. Junctional epithelial cells attach to each other only loosely, due to a low density of desmosomes and lack of E-cadherin. Thus, the intercellular space in the junctional epithelium is considerably more permeable than that of the sulcular and oral epithelia (9). Adhesion molecule-intracellular adhesion molecule-1 (17) and a potent chemokine for polymorphonuclear neutrophils, interleukin-8, (110) are expressed in the junctional epithelium. Expression is especially high in its superficial layer (111). This expression and the permeability of the junctional epithelium contribute to the constant recruitment and migration of polymorphonuclear neutrophils from sub-epithelial connective tissue blood vessels through the junctional epithelium into the gingival sulcus (111).

During migration, activated polymorphonuclear neutrophils release human neutrophil peptides, which in turn induce the release of epithelial interleukin-8 (93, 114), thus providing a local chemokine network. In human gingival crevicular fluid, the estimated concentration of polymorphonuclear neutrophils is about 800 000 per ml (99). These migratory polymorphonuclear neutrophils limit the growth and accumulation of dental plaque. The dynamic sequence described above results from cross-talk between resident non-immune cells and professional phagocytic cells, all of which participate in local innate immune activation.

Gingival crevicular fluid occurs in minute amounts, and is a complex mixture of substances derived from serum, leukocytes, structural cells of the periodontium and plaque bacteria (112). Detailed analyzes have shown the presence of serum albumin (81), lactoferrin (27), lysozyme (27), human neutrophil peptides (81, 83), human β-defensins (21), cathelicidin (LL37) (83), secretory leukocyte protease inhibitor (45), cystatin A (81), statherin (81) and other unidentified components (81). In terms of periodontal health, the presence of human neutrophil peptides, human β-defensins, cathelicidin (LL37), secretory leukocyte protease inhibitor, lactoferrin and cystatin in gingival crevicular fluid suggests an ongoing innate antibacterial and antiviral process. Human neutrophil peptides are produced by polymorphonuclear neutrophils (28, 86). Cathelicidin (LL37) is produced by both polymorphonuclear neutrophils and epithelial cells (121), and human β-defensins are produced only by epithelial cells (29).

The oral mucosal epithelium appears to be naturally resistant to viral infection. Recent reports have found constitutive expression of mucosal antiviral protein secretory leukocyte protease inhibitor in the oral epithelial cells of both healthy (46) and inflamed periodontal tissues (45). The levels of secretory leukocyte protease inhibitor significantly increase after *in vitro* exposure of gingival epithelial cells to HIV (46). Our group has detected expression of myxovirus resistance protein A (MxA) in healthy gingival tissue, especially in the junctional and sulcular epithelium (Fig. 3A) (unpublished data). This protein, a product of the human *MX1* gene, is induced by type I interferon and plays a critical role in immune responses against various viruses (35, 89). MxA is expressed in the cytoplasm and exhibits intrinsic antiviral activity. Interestingly we also found that human neutrophil peptides 1, 2 and 3, but not human β-defensins-1, -2 or -3 or cathelicidin (LL37), are able to induce MxA expression in human gingival epithelial cells (Fig. 3 B). Levels of human neutrophil peptides in the mg/ml range were observed in periodontal health gingival crevicular fluid (57, 68). These levels are within the lethal dose for key periodontal pathogens (71), and inhibit viral infectivity (22). These observations also suggest a role for active innate immune mechanisms in maintaining microbiota and modulating host cell induction of antiviral defense for periodontal homeostasis (Fig. 4).

Conclusions

The oral mucosa is important in tooth support, mastication, gustatory function and speech. Saliva moistens and lubricates the oral mucosa. In addition to these familiar functions, recent data have suggested a critical role for the oral mucosa and saliva in the protective innate immune response against viral

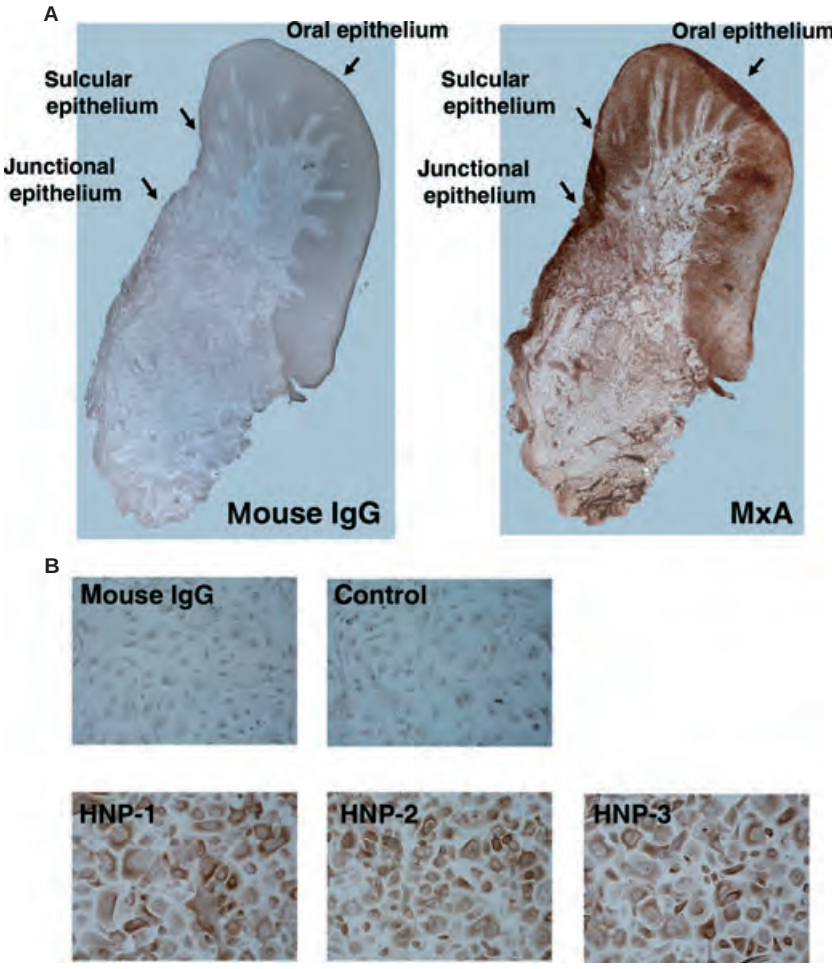

Fig. 3. Innate antiviral activity in periodontal tissues. (A). MxA expression in healthy gingiva. (B) HNP-induced MxA expression in human gingival epithelial cells. HNP, human neutrophil peptide; MxA, myxovirus resistance protein A.

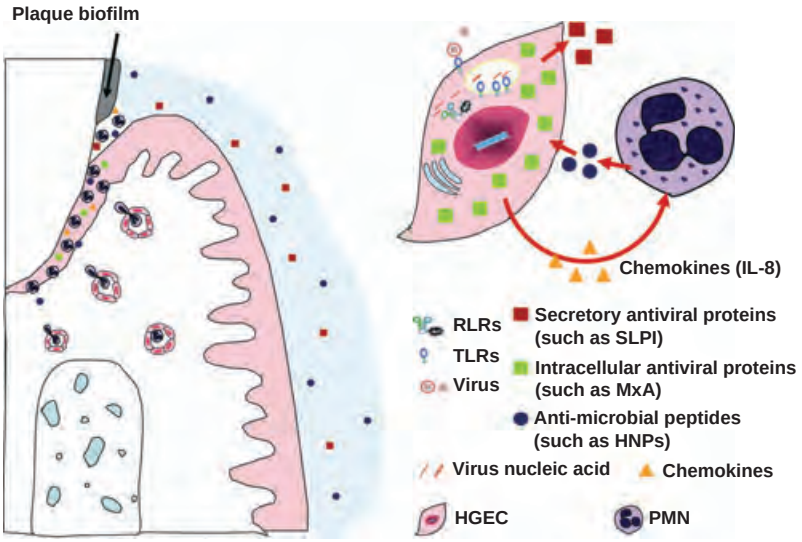

Fig. 4. Hypothetical diagram of antiviral innate immunity at the gingival sulcus area. Human gingival epithelial cells express pattern recognition receptors that recognize viral invasion. Continued migration of polymorphonuclear neutrophils across junctional epithelium leads to constant cross-talk between epithelial cells and migratory polymorphonuclear neutrophils. Antimicrobial peptides released from polymorphonuclear neutrophils, such as human neutrophil peptides, could up-regulate expression of antiviral MxA protein in gingival epithelium. HGEC, human gingival epithelial cells; HNP, human neutrophil peptides; IL-8, interleukin-8; MxA, myxovirus resistance protein A; PMN, polymorphonuclear neutrophils; RIG-I, retinoic acid-indcible gene I; RLRs, RIG-I-like receptors; TLRs, Toll-like receptors; SLPI, secretory leukocyte protease inhibitor.

infection. The presence in periodontal cells of TLRs and RLRs that are known to recognize viral envelopes and nucleic acids, coupled with the antiviral activity of secretory proteins in saliva and gingival crevicular fluid, indicate efficient antiviral innate immunity in the oral cavity. Expression of gingival interleukin-8 and MxA, which is possibly induced by polymorphonuclear neutrophil-derived human neutrophil peptides, suggests cross-talk between epithelial cells and polymorphonuclear neutrophils, especially in the gingival sulcus area. This further supports a role for periodontal tissue in innate immunity against viral infection. Further research is required to extend our understanding of the role of the periodontal epithelium in innate immune defense against viral infection, especially at the gingival sulcus. Research is also required to elucidate the role of innate immunity in maintaining periodontal homeostasis against constant challenge by commensal oral microorganisms.

Acknowledgments

We thank Dr Kitti Torrungruang (Department of Microbiology, Chulalongkorn University, Bangkok, Thailand) for constructive editorial comments. We also thank the Thailand Research Fund, the National Research Council of Thailand and the Ratchadapisek Endowment.

References

1. Andersen JH, Osbakk SA, Vorland LH, Traavik T, Gutteberg TJ. Lactoferrin and cyclic lactoferricin inhibit the entry of human cytomegalovirus into human fibroblasts. *Antiviral Res* 2001: **51**: 141–149.
2. Aravalli RN, Hu S, Lokensgard JR. Toll-like receptor 2 signaling is a mediator of apoptosis in herpes simplex virus-infected microglia. *J Neuroinflammation* 2007: **4**: 11.
3. Arduino PG, Porter SR. Herpes simplex virus type 1 infection: overview on relevant clinico-pathological features. *J Oral Pathol Med* 2008: **37**: 107–121.
4. Beignon AS, McKenna K, Skoberne M, Manches O, DaSilva I, Kavanagh DG, Larsson M, Gorelick RJ, Lifson JD, Bhardwaj N. Endocytosis of HIV-1 activates plasmacytoid dendritic cells via Toll-like receptor–viral RNA interactions. *J Clin Invest* 2005: **115**: 3265–3275.
5. Beklen A, Hukkanen M, Richardson R, Konttinen YT. Immunohistochemical localization of Toll-like receptors 1–10 in periodontitis. *Oral Microbiol Immunol* 2008: **23**: 425–431.
6. Beppu Y, Imamura Y, Tashiro M, Towatari T, Ariga H, Kido H. Human mucus protease inhibitor in airway fluids is a potential defensive compound against infection with influenza A and Sendai viruses. *J Biochem* 1997: **121**: 309–316.
7. Bergman P, Walter-Jallow L, Broliden K, Agerberth B, Soderlund J. The antimicrobial peptide LL-37 inhibits HIV-1 replication. *Curr HIV Res* 2007: **5**: 410–415.
8. Boehme KW, Guerrero M, Compton T. Human cytomegalovirus envelope glycoproteins B and H are necessary for TLR2 activation in permissive cells. *J Immunol* 2006: **177**: 7094–7102.
9. Bosshardt DD, Lang NP. The junctional epithelium: from health to disease. *J Dent Res* 2005: **84**: 9–20.
10. Buck CB, Day PM, Thompson CD, Lubkowski J, Lu W, Lowy DR, Schiller JT. Human α-defensins block papillomavirus infection. *Proc Natl Acad Sci USA* 2006: **103**: 1516–1521.
11. Chang TL, Francois F, Mosoian A, Klotman ME. CAF-mediated human immunodeficiency virus (HIV) type 1 transcriptional inhibition is distinct from α-defensin-1 HIV inhibition. *J Virol* 2003: **77**: 6777–6784.
12. Chang TL, Vargas J Jr, DelPortillo A, Klotman ME. Dual role of α-defensin-1 in anti-HIV-1 innate immunity. *J Clin Invest* 2005: **115**: 765–773.
13. Contreras A, Nowzari H, Slots J. Herpesviruses in periodontal pocket and gingival tissue specimens. *Oral Microbiol Immunol* 2000: **15**: 15–18.
14. Contreras A, Slots J. Mammalian viruses in human periodontitis. *Oral Microbiol Immunol* 1996: **11**: 381–386.
15. Contreras A, Slots J. Active cytomegalovirus infection in human periodontitis. *Oral Microbiol Immunol* 1998: **13**: 225–230.
16. Contreras A, Zadeh HH, Nowzari H, Slots J. Herpesvirus infection of inflammatory cells in human periodontitis. *Oral Microbiol Immunol* 1999: **14**: 206–212.
17. Crawford JM, Hopp B. Junctional epithelium expresses the intercellular adhesion molecule ICAM-1. *J Periodontal Res* 1990: **25**: 254–256.
18. Crombie R, Silverstein RL, MacLow C, Pearce SF, Nachman RL, Laurence J. Identification of a CD36-related thrombospondin 1-binding domain in HIV-1 envelope glycoprotein gp120: relationship to HIV-1-specific inhibitory factors in human saliva. *J Exp Med* 1998: **187**: 25–35.
19. Daher KA, Selsted ME, Lehrer RI. Direct inactivation of viruses by human granulocyte defensins. *J Virol* 1986: **60**: 1068–1074.
20. Dahlén G. Non-odontogenic infections in dentistry. *Periodontol 2000* 2009: **49**: 7–12.
21. Diamond DL, Kimball JR, Krisanaprakornkit S, Ganz T, Dale BA. Detection of β-defensins secreted by human oral epithelial cells. *J Immunol Methods* 2001: **256**: 65–76.
22. Doss M, White MR, Tecle T, Gantz D, Crouch EC, Jung G, Ruchala P, Waring AJ, Lehrer RI, Hartshorn KL. Interactions of α-, β-, and θ-defensins with influenza A virus and surfactant protein D. *J Immunol* 2009: **182**: 7878–7887.
23. Drobni P, Naslund J, Evander M. Lactoferrin inhibits human papillomavirus binding and uptake *in vitro*. *Antiviral Res* 2004: **64**: 63–68.
24. Ehrlich J, Cohen GH, Hochman N. Specific herpes simplex virus antigen in human gingiva. *J Periodontol* 1983: **54**: 357–360.
25. Fiorellini JP, Kim DM, Ishikawa SO. The gingiva. In: Newman MG, Takei H, Klokkevold PR, Carranza FA editors. *Carranza's clinical periodontology*, 10th edn. St. Louis, Missouri: Elsevier Health Sciences, 2006: 46–67.

26. Friedman HM. Keratin, a dual role in herpes simplex virus pathogenesis. *J Clin Virol* 2006: **35**: 103–105.
27. Friedman SA, Mandel ID, Herrera MS. Lysozyme and lactoferrin quantitation in the crevicular fluid. *J Periodontol* 1983: **54**: 347–350.
28. Ganz T. Extracellular release of antimicrobial defensins by human polymorphonuclear leukocytes. *Infect Immun* 1987: **55**: 568–571.
29. Ganz T. Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol* 2003: **3**: 710–720.
30. Gargiulo A, Wentz F, Orban B. Dimensions and relations of the dento-gingival junction in humans. *J Periodontol* 1961: **32**: 261–267.
31. Gaudreault E, Fiola S, Olivier M, Gosselin J. Epstein–Barr virus induces MCP-1 secretion by human monocytes via TLR2. *J Virol* 2007: **81**: 8016–8024.
32. Goebel C, Mackay LG, Vickers ER, Mather LE. Determination of defensin HNP-1, HNP-2, and HNP-3 in human saliva by using LC/MS. *Peptides* 2000: **21**: 757–765.
33. Guggemoos S, Hangel D, Hamm S, Heit A, Bauer S, Adler H. TLR9 contributes to antiviral immunity during gammaherpesvirus infection. *J Immunol* 2008: **180**: 438–443.
34. Haffajee AD, Socransky SS. Microbial etiological agents of destructive periodontal diseases. *Periodontol 2000* 1994: **5**: 78–111.
35. Haller O, Staeheli P, Kochs G. Interferon-induced Mx proteins in antiviral host defense. *Biochimie* 2007: **89**: 812–818.
36. Hardestam J, Petterson L, Ahlm C, Evander M, Lundkvist A, Klingstrom J. Antiviral effect of human saliva against hantavirus. *J Med Virol* 2008: **80**: 2122–2126.
37. Harmsen MC, Swart PJ, de Bethune MP, Pauwels R, De Clercq E, The TH, Meijer DK. Antiviral effects of plasma and milk proteins: lactoferrin shows potent activity against both human immunodeficiency virus and human cytomegalovirus replication *in vitro*. *J Infect Dis* 1995: **172**: 380–388.
38. Hasegawa K, Motsuchi W, Tanaka S, Dosako S. Inhibition with lactoferrin of *in vitro* infection with human herpes virus. *Jpn J Med Sci Biol* 1994: **47**: 73–85.
39. Hazrati E, Galen B, Lu W, Wang W, Ouyang Y, Keller MJ, Lehrer RI, Herold BC. Human α- and β-defensins block multiple steps in herpes simplex virus infection. *J Immunol* 2006: **177**: 8658–8666.
40. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* 2004: **303**: 1526–1529.
41. Hochrein H, Schlatter B, O'Keeffe M, Wagner C, Schmitz F, Schiemann M, Bauer S, Suter M, Wagner H. Herpes simplex virus type-1 induces IFN-α production via Toll-like receptor 9-dependent and -independent pathways. *Proc Natl Acad Sci USA* 2004: **101**: 11416–11421.
42. Holt SC, Ebersole JL. *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*: the 'red complex', a prototype polybacterial pathogenic consortium in periodontitis. *Periodontol 2000* 2005: **38**: 72–122.
43. Honda K, Takaoka A, Taniguchi T. Type I interferon gene induction by the interferon regulatory factor family of transcription factors. *Immunity* 2006: **25**: 349–360.
44. Ikeda M, Nozaki A, Sugiyama K, Tanaka T, Naganuma A, Tanaka K, Sekihara H, Shimotohno K, Saito M, Kato N. Characterization of antiviral activity of lactoferrin against hepatitis C virus infection in human cultured cells. *Virus Res* 2000: **66**: 51–63.
45. Into T, Inomata M, Kanno Y, Matsuyama T, Machigashira M, Izumi Y, Imamura T, Nakashima M, Noguchi T, Matsushita K. Arginine-specific gingipains from *Porphyromonas gingivalis* deprive protective functions of secretory leucocyte protease inhibitor in periodontal tissue. *Clin Exp Immunol* 2006: **145**: 545–554.
46. Jana NK, Gray LR, Shugars DC. Human immunodeficiency virus type 1 stimulates the expression and production of secretory leukocyte protease inhibitor (SLPI) in oral epithelial cells: a role for SLPI in innate mucosal immunity. *J Virol* 2005: **79**: 6432–6440.
47. John M, Keller MJ, Fam EH, Cheshenko N, Hogarty K, Kasowitz A, Wallenstein S, Carlucci MJ, Tuyama AC, Lu W, Klotman ME, Lehrer RI, Herold BC. Cervicovaginal secretions contribute to innate resistance to herpes simplex virus infection. *J Infect Dis* 2005: **192**: 1731–1740.
48. Kanneganti TD, Body-Malapel M, Amer A, Park JH, Whitfield J, Franchi L, Taraporewala ZF, Miller D, Patton JT, Inohara N, Nunez G. Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. *J Biol Chem* 2006: **281**: 36560–36568.
49. Kawai T, Akira S. Toll-like receptor and RIG-I-like receptor signaling. *Ann NY Acad Sci* 2008: **1143**: 1–20.
50. Kawai T, Akira S. The roles of TLRs, RLRs and NLRs in pathogen recognition. *Int Immunol* 2009: **21**: 317–337.
51. Krug A, Luker GD, Barchet W, Leib DA, Akira S, Colonna M. Herpes simplex virus type 1 activates murine natural interferon-producing cells through toll-like receptor 9. *Blood* 2004: **103**: 1433–1437.
52. Kubar A, Saygun I, Yapar M, Özdemir A, Slots J. Real-time PCR quantification of cytomegalovirus in aggressive periodontitis lesions using TaqMan technology. *J Periodontal Res* 2004: **39**: 81–86.
53. Kubota K, Sakaki H, Imaizumi T, Nakagawa H, Kusumi A, Kobayashi W, Satoh K, Kimura H. Retinoic acid-inducible gene-I is induced in gingival fibroblasts by lipopolysaccharide or poly IC: possible roles in interleukin-1β, -6 and -8 expression. *Oral Microbiol Immunol* 2006: **21**: 399–406.
54. Kurt-Jones EA, Chan M, Zhou S, Wang J, Reed G, Bronson R, Arnold MM, Knipe DM, Finberg RW. Herpes simplex virus 1 interaction with Toll-like receptor 2 contributes to lethal encephalitis. *Proc Natl Acad Sci USA* 2004: **101**: 1315–1320.
55. Latz E, Schoenemeyer A, Visintin A, Fitzgerald KA, Monks BG, Knetter CF, Lien E, Nilsen NJ, Espevik T, Golenbock DT. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat Immunol* 2004: **5**: 190–198.
56. Lund J, Sato A, Akira S, Medzhitov R, Iwasaki A. Toll-like receptor 9-mediated recognition of herpes simplex virus-2 by plasmacytoid dendritic cells. *J Exp Med* 2003: **198**: 513–520.
57. Lundy FT, Orr DF, Shaw C, Lamey PJ, Linden GJ. Detection of individual human neutrophil α-defensins (human neutrophil peptides 1, 2 and 3) in unfractionated gingival crevicular fluid – a MALDI-MS approach. *Mol Immunol* 2005: **42**: 575–579.
58. Mackewicz CE, Yuan J, Tran P, Diaz L, Mack E, Selsted ME, Levy JA. α-Defensins can have anti-HIV activity but are not CD8 cell anti-HIV factors. *AIDS* 2003: **17**: F23–F32.

59. Madinier I, Doglio A, Cagnon L, Lefebvre JC, Monteil RA. Southern blot detection of human papillomaviruses (HPVs) DNA sequences in gingival tissues. *J Periodontol* 1992: **63**: 667–673.
60. Mahanonda R, Pichyangkul S. Toll-like receptors and their role in periodontal health and disease. *Periodontol 2000* 2007: **43**: 41–55.
61. Mahanonda R, Sa-Ard-Iam N, Eksomtramate M, Rerkyen P, Phairat B, Schaecher KE, Fukuda MM, Pichyangkul S. Cigarette smoke extract modulates human β-defensin-2 and interleukin-8 expression in human gingival epithelial cells. *J Periodontal Res* 2009: **44**: 557–564.
62. Mahanonda R, Sa-Ard-Iam N, Montreekachon P, Pimkhaokham A, Yongvanichit K, Fukuda MM, Pichyangkul S. IL-8 and IDO expression by human gingival fibroblasts via TLRs. *J Immunol* 2007: **178**: 1151–1157.
63. Marchetti M, Ammendolia MG, Superti F. Glycosaminoglycans are not indispensable for the anti-herpes simplex virus type 2 activity of lactoferrin. *Biochimie* 2009: **91**: 155–159.
64. Marchetti M, Superti F, Ammendolia MG, Rossi P, Valenti P, Seganti L. Inhibition of poliovirus type 1 infection by iron-, manganese- and zinc-saturated lactoferrin. *Med Microbiol Immunol* 1999: **187**: 199–204.
65. Marchetti M, Trybala E, Superti F, Johansson M, Bergstrom T. Inhibition of herpes simplex virus infection by lactoferrin is dependent on interference with the virus binding to glycosaminoglycans. *Virology* 2004: **318**: 405–413.
66. Mathews M, Jia HP, Guthmiller JM, Losh G, Graham S, Johnson GK, Tack BF, McCray PB Jr. Production of β-defensin antimicrobial peptides by the oral mucosa and salivary glands. *Infect Immun* 1999: **67**: 2740–2745.
67. Maticic M, Poljak M, Kramar B, Seme K, Brinovec V, Meglic-Volkar J, Zakotnik B, Skaleric U. Detection of hepatitis C virus RNA from gingival crevicular fluid and its relation to virus presence in saliva. *J Periodontol* 2001: **72**: 11–16.
68. McKay MS, Olson E, Hesla MA, Panyutich A, Ganz T, Perkins S, Rossomando EF. Immunomagnetic recovery of human neutrophil defensins from the human gingival crevice. *Oral Microbiol Immunol* 1999: **14**: 190–193.
69. McNeely TB, Dealy M, Dripps DJ, Orenstein JM, Eisenberg SP, Wahl SM. Secretory leukocyte protease inhibitor: a human saliva protein exhibiting anti-human immunodeficiency virus 1 activity *in vitro*. *J Clin Invest* 1995: **96**: 456–464.
70. Meyer DH, Fives-Taylor PM. The role of *Actinobacillus actinomycetemcomitans* in the pathogenesis of periodontal disease. *Trends Microbiol* 1997: **5**: 224–228.
71. Miyasaki KT, Bodeau AL, Selsted ME, Ganz T, Lehrer RI. Killing of oral, gram-negative, facultative bacteria by the rabbit defensin, NP-1. *Oral Microbiol Immunol* 1990: **5**: 315–319.
72. Mocarski EJ, Shenk T, Pass R. Cytomegaloviruses. In: Knipe DM, Howley PM editors. *Fields virology*, 5th edn. Philadelphia, PA: Lippincott Williams & Wilkins, 2007: 2702–2772.
73. Murakami M, Ohtake T, Dorschner RA, Gallo RL. Cathelicidin antimicrobial peptides are expressed in salivary glands and saliva. *J Dent Res* 2002: **81**: 845–850.
74. Nishiya T, Kajita E, Miwa S, Defranco AL. TLR3 and TLR7 are targeted to the same intracellular compartments by distinct regulatory elements. *J Biol Chem* 2005: **280**: 37107–37117.
75. Nozaki A, Ikeda M, Naganuma A, Nakamura T, Inudoh M, Tanaka K, Kato N. Identification of a lactoferrin-derived peptide possessing binding activity to hepatitis C virus E2 envelope protein. *J Biol Chem* 2003: **278**: 10162–10173.
76. Oppenheim FG, Salih E, Siqueira WL, Zhang W, Helmerhorst EJ. Salivary proteome and its genetic polymorphisms. *Ann NY Acad Sci* 2007: **1098**: 22–50.
77. Parra B, Slots J. Detection of human viruses in periodontal pockets using polymerase chain reaction. *Oral Microbiol Immunol* 1996: **11**: 289–293.
78. Pass RF. Cytomegalovirus infection. *Pediatr Rev* 2002: **23**: 163–170.
79. Paster BJ, Olsen I, Aas JA, Dewhirst FE. The breadth of bacterial diversity in the human periodontal pocket and other oral sites. *Periodontol 2000* 2006: **42**: 80–87.
80. Pichlmair A, Reis e Sousa C. Innate recognition of viruses. *Immunity* 2007: **27**: 370–383.
81. Pisano E, Cabras T, Montaldo C, Piras V, Inzitari R, Olmi C, Castagnola M, Messana I. Peptides of human gingival crevicular fluid determined by HPLC-ESI-MS. *Eur J Oral Sci* 2005: **113**: 462–468.
82. Puddu P, Borghi P, Gessani S, Valenti P, Belardelli F, Seganti L. Antiviral effect of bovine lactoferrin saturated with metal ions on early steps of human immunodeficiency virus type 1 infection. *Int J Biochem Cell Biol* 1998: **30**: 1055–1062.
83. Puklo M, Guentsch A, Hiemstra PS, Eick S, Potempa J. Analysis of neutrophil-derived antimicrobial peptides in gingival crevicular fluid suggests importance of cathelicidin LL-37 in the innate immune response against periodontogenic bacteria. *Oral Microbiol Immunol* 2008: **23**: 328–335.
84. Py B, Basmaciogullari S, Bouchet J, Zarka M, Moura IC, Benhamou M, Monteiro RC, Hocini H, Madrid R, Benichou S. The phospholipid scramblases 1 and 4 are cellular receptors for the secretory leukocyte protease inhibitor and interact with CD4 at the plasma membrane. *PLoS ONE* 2009: **4**: e5006.
85. Quiñones-Mateu ME, Lederman MM, Feng Z, Chakraborty B, Weber J, Rangel HR, Marotta ML, Mirza M, Jiang B, Kiser P, Medvik K, Sieg SF, Weinberg A. Human epithelial β-defensins 2 and 3 inhibit HIV-1 replication. *AIDS* 2003: **17**: F39–F48.
86. Rice WG, Ganz T, Kinkade JM Jr, Selsted ME, Lehrer RI, Parmley RT. Defensin-rich dense granules of human neutrophils. *Blood* 1987: **70**: 757–765.
87. Robinovitch MR, Ashley RL, Iversen JM, Vigoren EM, Oppenheim FG, Lamkin M. Parotid salivary basic proline-rich proteins inhibit HIV-1 infectivity. *Oral Dis* 2001: **7**: 86–93.
88. Roizman B, Knipe DM, Whitley R. Herpes simplex viruses. In: Knipe DM, Howley PM editors. *Fields virology*, 5th edn. Philadelphia, PA: Lippincott Williams & Wilkins, 2007: 2502–2601.
89. Ronni T, Melen K, Malygin A, Julkunen I. Control of IFN-inducible MxA gene expression in human cells. *J Immunol* 1993: **150**: 1715–1726.
90. Rothenfusser S, Goutagny N, DiPerna G, Gong M, Monks BG, Schoenemeyer A, Yamamoto M, Akira S, Fitzgerald KA. The RNA helicase Lgp2 inhibits TLR-independent sensing

of viral replication by retinoic acid-inducible gene-I. *J Immunol* 2005: **175**: 5260–5268.
91. Saboia-Dantas CJ, Coutrin de Toledo LF, Sampaio-Filho HR, Siqueira JF Jr. Herpesviruses in asymptomatic apical periodontitis lesions: an immunohistochemical approach. *Oral Microbiol Immunol* 2007: **22**: 320–325.
92. Sakaki H, Imaizumi T, Matsumiya T, Kusumi A, Nakagawa H, Kubota K, Nishi N, Nakamura T, Hirashima M, Satoh K, Kimura H. Retinoic acid-inducible gene-I is induced by interleukin-1β in cultured human gingival fibroblasts. *Oral Microbiol Immunol* 2005: **20**: 47–50.
93. Sakamoto N, Mukae H, Fujii T, Ishii H, Yoshioka S, Kakugawa T, Sugiyama K, Mizuta Y, Kadota J, Nakazato M, Kohno S. Differential effects of α- and β-defensin on cytokine production by cultured human bronchial epithelial cells. *Am J Physiol* 2005: **288**: L508–L513.
94. Sano H, Nagai K, Tsutsumi H, Kuroki Y. Lactoferrin and surfactant protein A exhibit distinct binding specificity to F protein and differently modulate respiratory syncytial virus infection. *Eur J Immunol* 2003: **33**: 2894–2902.
95. Sato A, Linehan MM, Iwasaki A. Dual recognition of herpes simplex viruses by TLR2 and TLR9 in dendritic cells. *Proc Natl Acad Sci USA* 2006: **103**: 17343–17348.
96. Saygun I, Sahin S, Özdemir A, Kurtis B, Yapar M, Kubar A, Ozcan G. Detection of human viruses in patients with chronic periodontitis and the relationship between viruses and clinical parameters. *J Periodontol* 2002: **73**: 1437–1443.
97. Saygun I, Yapar M, Özdemir A, Kubar A, Slots J. Human cytomegalovirus and Epstein–Barr virus type 1 in periodontal abscesses. *Oral Microbiol Immunol* 2004: **19**: 83–87.
98. Schenkein HA. Host responses in maintaining periodontal health and determining periodontal disease. *Periodontol 2000* 2006: **40**: 77–93.
99. Skapski H, Lehner T. A crevicular washing method for investigating immune components of crevicular fluid in man. *J Periodontal Res* 1976: **11**: 19–24.
100. Skott P, Lucht E, Ehnlund M, Bjorling E. Inhibitory function of secretory leukocyte proteinase inhibitor (SLPI) in human saliva is HIV-1 specific and varies with virus tropism. *Oral Dis* 2002: **8**: 160–167.
101. Skougaard MR. Cell renewal, with special reference to the gingival epithelium. *Adv Oral Biol* 1970: **4**: 261–288.
102. Slots J. *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in periodontal disease: introduction. *Periodontol 2000* 1999: **20**: 7–13.
103. Slots J. Human viruses in periodontitis. *Periodontol 2000* 2010: **53**: 89–110.
104. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol* 1998: **25**: 134–144.
105. Tabeta K, Georgel P, Janssen E, Du X, Hoebe K, Crozat K, Mudd S, Shamel L, Sovath S, Goode J, Alexopoulou L, Flavell RA, Beutler B. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proc Natl Acad Sci USA* 2004: **101**: 3516–3521.
106. Takaoka A, Wang Z, Choi MK, Yanai H, Negishi H, Ban T, Lu Y, Miyagishi M, Kodama T, Honda K, Ohba Y, Taniguchi T. DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. *Nature* 2007: **448**: 501–505.
107. Teng Y-TA. Protective and destructive immunity in the periodontium: part 1 – innate and humoral immunity and the periodontium. *J Dent Res* 2006: **85**: 198–208.
108. Theofilopoulos AN, Baccala R, Beutler B, Kono DH. Type I interferons (α/β) in immunity and autoimmunity. *Annu Rev Immunol* 2005: **23**: 307–336.
109. Ting M, Contreras A, Slots J. Herpesvirus in localized juvenile periodontitis. *J Periodontal Res* 2000: **35**: 17–25.
110. Tonetti MS. Molecular factors associated with compartmentalization of gingival immune responses and transepithelial neutrophil migration. *J Periodontal Res* 1997: **32**: 104–109.
111. Tonetti MS, Imboden MA, Lang NP. Neutrophil migration into the gingival sulcus is associated with transepithelial gradients of interleukin-8 and ICAM-1. *J Periodontol* 1998: **69**: 1139–1147.
112. Uitto VJ. Gingival crevice fluid – an introduction. *Periodontol 2000* 2003: **31**: 9–11.
113. Valimaa H, Tenovuo J, Waris M, Hukkanen V. Human lactoferrin but not lysozyme neutralizes HSV-1 and inhibits HSV-1 replication and cell-to-cell spread. *Virol J* 2009: **6**: 53.
114. Van Wetering S, Mannesse-Lazeroms SP, Van Sterkenburg MA, Daha MR, Dijkman JH, Hiemstra PS. Effect of defensins on interleukin-8 synthesis in airway epithelial cells. *Am J Physiol* 1997: **272**: L888–L896.
115. Wahl SM, McNeely TB, Janoff EN, Shugars D, Worley P, Tucker C, Orenstein JM. Secretory leukocyte protease inhibitor (SLPI) in mucosal fluids inhibits HIV-1. *Oral Dis* 1997: **3**(Suppl. 1): S64–S69.
116. White MR, Helmerhorst EJ, Ligtenberg A, Karpel M, Tecle T, Siqueira WL, Oppenheim FG, Hartshorn KL. Multiple components contribute to ability of saliva to inhibit influenza viruses. *Oral Microbiol Immunol* 2009: **24**: 18–24.
117. Williams H, Crawford DH. Epstein–Barr virus: the impact of scientific advances on clinical practice. *Blood* 2006: **107**: 862–869.
118. Yapar M, Saygun I, Ozdemir A, Kubar A, Sahin S. Prevalence of human herpesviruses in patients with aggressive periodontitis. *J Periodontol* 2003: **74**: 1634–1640.
119. Yoneyama M, Kikuchi M, Matsumoto K, Imaizumi T, Miyagishi M, Taira K, Foy E, Loo YM, Gale M Jr, Akira S, Yonehara S, Kato A, Fujita T. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J Immunol* 2005: **175**: 2851–2858.
120. Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* 2004: **5**: 730–737.
121. Zanetti M. Cathelicidins, multifunctional peptides of the innate immunity. *J Leukoc Biol* 2004: **75**: 39–48.
122. Zasloff M. Innate immunity, antimicrobial peptides, and protection of the oral cavity. *Lancet* 2002: **360**: 1116–1117.
123. Zhang L, Yu W, He T, Yu J, Caffrey RE, Dalmasso EA, Fu S, Pham T, Mei J, Ho JJ, Zhang W, Lopez P, Ho DD. Contribution of human α-defensin 1, 2, and 3 to the anti-HIV-1 activity of CD8 antiviral factor. *Science* 2002: **298**: 995–1000. Retracted in *Science* 2004: **303:** 467.

Reprint form European Journal of Immunology

 DOI: 10.1002/eji.201141657 Eur. J. Immunol. 2012. 42: 946–956

European Journal of Immunology

MxA expression induced by α-defensin in healthy human periodontal tissue

Rangsini Mahanonda[1,2], Noppadol Sa-Ard-Iam[2], Pimprapa Rerkyen[2], Arunee Thitithanyanont[3], Keskanya Subbalekha[4] and Sathit Pichyangkul[5]

[1] Department of Periodontology Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand
[2] Research Unit for Periodontal Disease Immunology Laboratory Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand
[3] Department of Microbiology Faculty of Science, Mahidol University, Bangkok, Thailand
[4] Department of Oral Maxillofacial Surgery Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand
[5] Department of Immunology and Medicine US Army Medical Component, Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand

Although periodontal tissue is continually challenged by microbial plaque, it is generally maintained in a healthy state. To understand the basis for this, we investigated innate antiviral immunity in human periodontal tissue. The expression of mRNA encoding different antiviral proteins, myxovirus resistance A (MxA), protein kinase R (PKR), oligoadenylate synthetase (OAS), and secretory leukocyte protease inhibitor (SLPI) were detected in both healthy tissue and that with periodontitis. Immunostaining data consistently showed higher MxA protein expression in the epithelial layer of healthy gingiva as compared with tissue with periodontitis. Human MxA is thought to be induced by type I and III interferons (IFNs) but neither cytokine type was detected in healthy periodontal tissues. Treatment in vitro of primary human gingival epithelial cells (HGECs) with α-defensins, but not with the antimicrobial peptides β-defensins or LL-37, led to MxA protein expression. α-defensin was also detected in healthy periodontal tissue. In addition, MxA in α-defensin-treated HGECs was associated with protection against avian influenza H5N1 infection and silencing of the MxA gene using MxA-targeted-siRNA abolished this antiviral activity. To our knowledge, this is the first study to uncover a novel pathway of human MxA induction, which is initiated by an endogenous antimicrobial peptide, namely α-defensin. This pathway may play an important role in the first line of antiviral defense in periodontal tissue.

Keywords: α-defensin • Homeostasis • MxA • Periodontal tissue

Supporting Information available online

Correspondence: Dr. Rangsini Mahanonda
e-mail: rangsini.m@chula.ac.th

Introduction

Periodontal tissue is a tooth-supporting structure, which includes gingiva, periodontal ligaments, cementum, and alveolar bone. Chronic inflammation of the periodontal tissue, periodontal disease, is one of the most common inflammatory diseases in humans. The advanced form of the disease, periodontitis, with severe bone destruction may cause tooth loss. The etiologic importance of bacteria in periodontal disease has been well recognized. Bacterial plaque biofilms continually form on the tooth surfaces adjacent to gingiva. Recent studies have proposed that viral co-infection could enhance the development and progression of periodontitis [1, 2]. Detection of herpes simplex virus (HSV) types 1 and 2, human cytomegalovirus (CMV), Epstein-Barr virus (EBV), and human immunodeficiency virus (HIV), have been reported in dental plaque biofilm, gingival crevicular fluid, and periodontitis tissue specimens [3]. In healthy periodontal specimens, some viral deoxyribonucleic acid (DNA) can also be found, but generally at lower levels than in periodontitis [4–6]. Even so, the precise role of viruses in periodontal disease remains unclear.

Periodontal tissue is continually exposed to bacterial plaque; therefore an effective innate immune response is critical to maintain homeostasis. Several studies demonstrated that different cell types in periodontal tissues including gingival epithelium, fibroblasts, and osteoblasts are well equipped with a variety of pattern-recognition receptors such as Toll-like receptors (TLRs) [7–10]. Triggering of these TLRs in human gingival epithelial cells (HGECs) with their specific ligands leads to production of mediators such as IL-8 and antimicrobial β-defensin-2 [9], highlighting the critical role of periodontal tissue in innate immunity.

To date, there is relatively little available information regarding periodontal innate antiviral immunity. In addition to TLR expression, the gingival epithelium and gingival fibroblasts express retinoic acid-inducible gene (RIG)-like receptors (RLRs), including RIG-I and melanoma differentiation associated gene 5 (MDA5) (unpublished observation; [11, 12]) which recognize viral ssRNA and dsRNA. Activation via these RLRs results in expression of inflammatory cytokines and type I interferon (IFN) [13]. Type I IFN is a key mediator in defense against viral infection. It eliminates viruses by enhancing the transcription of many IFN-inducible genes such as myxovirus resistance A (MxA) [14]. It also enhances dendritic cell maturation, antibody production, and differentiation of virus-specific cytotoxic T lymphocytes, resulting in effective adaptive immunity against viral infection [15, 16].

Saliva and gingival crevicular fluids, which bathe the periodontal tissue, contain a variety of innate immune mediators against bacteria, including human α-defensins (commonly known as human neutrophil peptides) [17], β-defensins [18], cathelicidin (LL-37) [19], thrombospondins [20], lactoferrin [21], and secretory leukocyte protease inhibitor (SLPI) [21]. Some of these molecules have also demonstrated antiviral properties [22]. To further gain insight into innate antiviral immunity, we investigated expression of antiviral proteins in periodontal tissue focusing on MxA, a potent antiviral protein against both RNA and DNA viruses [23–25].

Results

mRNA expression of antiviral proteins in periodontal tissues

SLPI has been reported in relation to antiviral defense in periodontal tissue [26]. In this study, we evaluated the expression of other antiviral molecules, including MxA, oligoadenylate synthetase (OAS), and protein kinase R (PKR) from both healthy periodontal tissue and periodontitis specimens. Using real-time RT-PCR, we found mRNA expression of MxA, OAS, PKR, and SLPI in all examined periodontal tissues. As compared with healthy periodontal tissues, the mean fold increase of relative quantification of MxA, OAS, PKR, and SLPI in periodontitis tissues was 0.83 ± 0.24, 1.06 ± 0.30, 1.20 ± 0.34, and 2.74 ± 1.37, respectively (Fig. 1). These differences between healthy and periodontitis tissues were not statistically significant ($p > 0.05$).

MxA protein expression in periodontal tissues

MxA protein is well known to have antiviral activity against both RNA and DNA viruses [24, 25]. We focused on MxA protein throughout our study. Immunohistochemical data in Fig. 2A and 2B shows that MxA protein expression was clearly observed in the epithelial layer of periodontal tissue. Epithelial MxA immunoreactivity seemed to be stronger in basal and spinous layers than outermost layer of oral epithelium. Using semiquantitative scoring, there was a significantly higher score of epithelial MxA in healthy group than periodontitis group (Table 1) ($p = 0.012$), thus highlighting the role of MxA protein in healthy periodontal tissue.

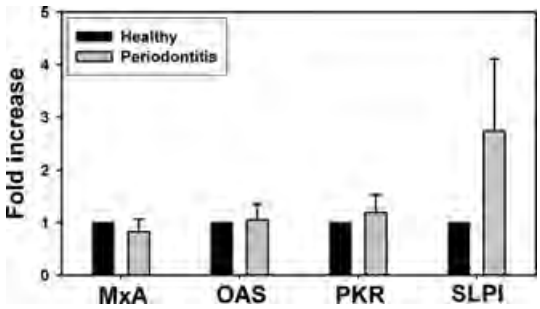

Figure 1. No differences in mRNA expression of antiviral proteins between healthy and periodontitis tissue. Periodontal tissue specimens were used for real-time RT-PCR for each of the indicated molecules as described in the *Materials and methods*. The relative quantification of mRNA expression for each antiviral protein in the periodontitis tissues was normalized to corresponding GAPDH, and presented as the mean fold increase + SEM, considering the mean value obtained from healthy periodontal tissues as a reference (relative quantification = 1) ($n = 10$ in each group).

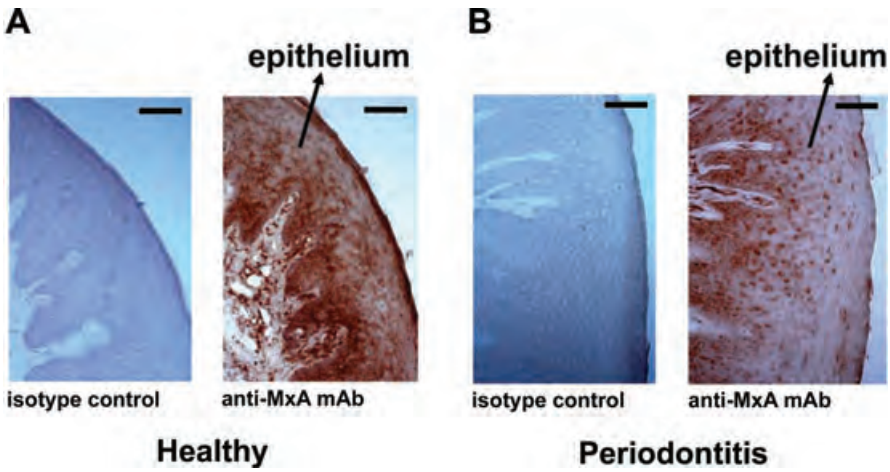

Figure 2. MxA protein expression in the epithelial layer and increased expression in healthy periodontal tissue. Frozen sections of (A) healthy periodontal tissues and (B) tissues with periodontitis were stained with either an anti-MxA antibody and isotype control by immunoperoxidase technique, and counterstained with hematoxylin. Data are representative of nine healthy periodontal tissues and seven periodontitis tissues. Each scale bar represents 100 μM.

Table 1. Immunoreactive staining score of MxA protein in epithelial layer of healthy ($n = 9$) and periodontitis ($n = 7$) tissue specimens.

Group	Immunoreactive staining score[a)]			
	0	1	2	3
	n (%)	*n* (%)	*n* (%)	*n* (%)
Healthy	0	0	0	9 (100)
Periodontitis	0	1 (14)	3 (43)	3 (43)

[a)] Score 1: the area of positive cells < 10%; score 2: 10–50%, and score 3: > 50%.

α-defensin-induced MxA protein expression in HGECs

Since MxA protein is known to be induced by type I and type III IFN [27–29], we then investigated the presence of type I and type III IFN in periodontal tissue. The mRNA expression of IFN-α, IFN-β, and IFN-λ in healthy periodontal tissue was negligible ($n = 10$, data not shown). The findings led us to hypothesize that other local mediators may be responsible for the observed MxA protein expression in healthy periodontal tissue.

Antimicrobial peptides including α-defensin, β-defensin, and LL-37 are constitutively expressed in healthy periodontal tissue [30] and these mediators could conceivably play a role in MxA expression. Furthermore, a recent study described a fish homologue of MxA protein which was induced by human α-defensin [31]. Therefore, we stimulated primary HGEC cultures with nontoxic concentrations of α-defensin-1, -2, and -3, β-defensin-1, -2, and -3, and LL-37. Fig. 3A shows that α-defensin-1, -2, and -3 markedly induced MxA protein in HGECs. There seemed to be stronger MxA staining in HGECs treated with α-defensin-1 than in those treated with α-defensin-2 and α-defensin-3. In contrast, β-defensin-1, -2, -3 and LL-37 induced only negligible MxA protein expression. IFN-α was used as positive control and induced strong MxA protein expression. The results of MxA protein expression induced by α-defensin-1, -2, and -3, β-defensin-1, -2, and -3, and LL-37 agree with mRNA expression using real-time RT-PCR (Fig. 3B). α-defensin-1 was also able to stimulate MxA protein expression in other cells including normal human bronchial epithelial cells and primary human microvascular endothelial cells (Fig. 3C).

Addition of neutralizing antibodies against type I IFN (IFN-α and IFN-β) into the cultures of α-defensin-1-treated HGECs had no effect on MxA expression whereas these neutralizing antibodies markedly inhibited MxA expression in IFN-α-treated HGECs (Fig. 3D). The IFN-α-induced MxA protein expression was likely to be independent on α-defensins since no detection of α-defensin production was observed in cultures of IFN-α-treated HGECs (Supporting Information Fig. 1). In addition, no production of type I IFN (IFN-α and IFN-β) was observed at both the mRNA and protein levels in α-defensin-treated HGECs (data not shown). Collectively, these data suggest that α-defensin and type I interferon use different triggering pathways to induce MxA expression.

Role of periodontal tissue MxA against influenza A viral infection

The antiviral activity of MxA against influenza A virus is well recognized [25]. Our preliminary data showed that unlike seasonal influenza virus, avian influenza H5N1 virus infected primary HGECs. We then employed H5N1 infection as a model to study the antiviral activity of α-defensin-induced MxA. The viral plaque assay in Fig. 4A shows that, similar to IFN-α-pretreated HGECs, α-defensin-1, -2, and -3-pretreated cells significantly inhibited H5N1 replication, suggesting a functional MxA protein. On the other hand, β-defensin-1, -2, -3, and LL-37-pretreated HGECs poorly inhibited viral replication. These findings were confirmed by microscopically observed cytopathic effects (data not shown).

To confirm the antiviral activity of MxA against H5N1, we transfected HGECs with MxA-targeted siRNA, treated the cells with α-defensin-1 overnight, and then infected them with H5N1 virus. MxA-targeted siRNA greatly reduced levels of MxA mRNA expression by 95%, (Fig. 4B) and effectively abolished inhibition of viral replication by 93% in H5N1-infected HGECs (Fig. 4C). These findings were supported by microscopically observed cytopathic effects (Fig. 4D).

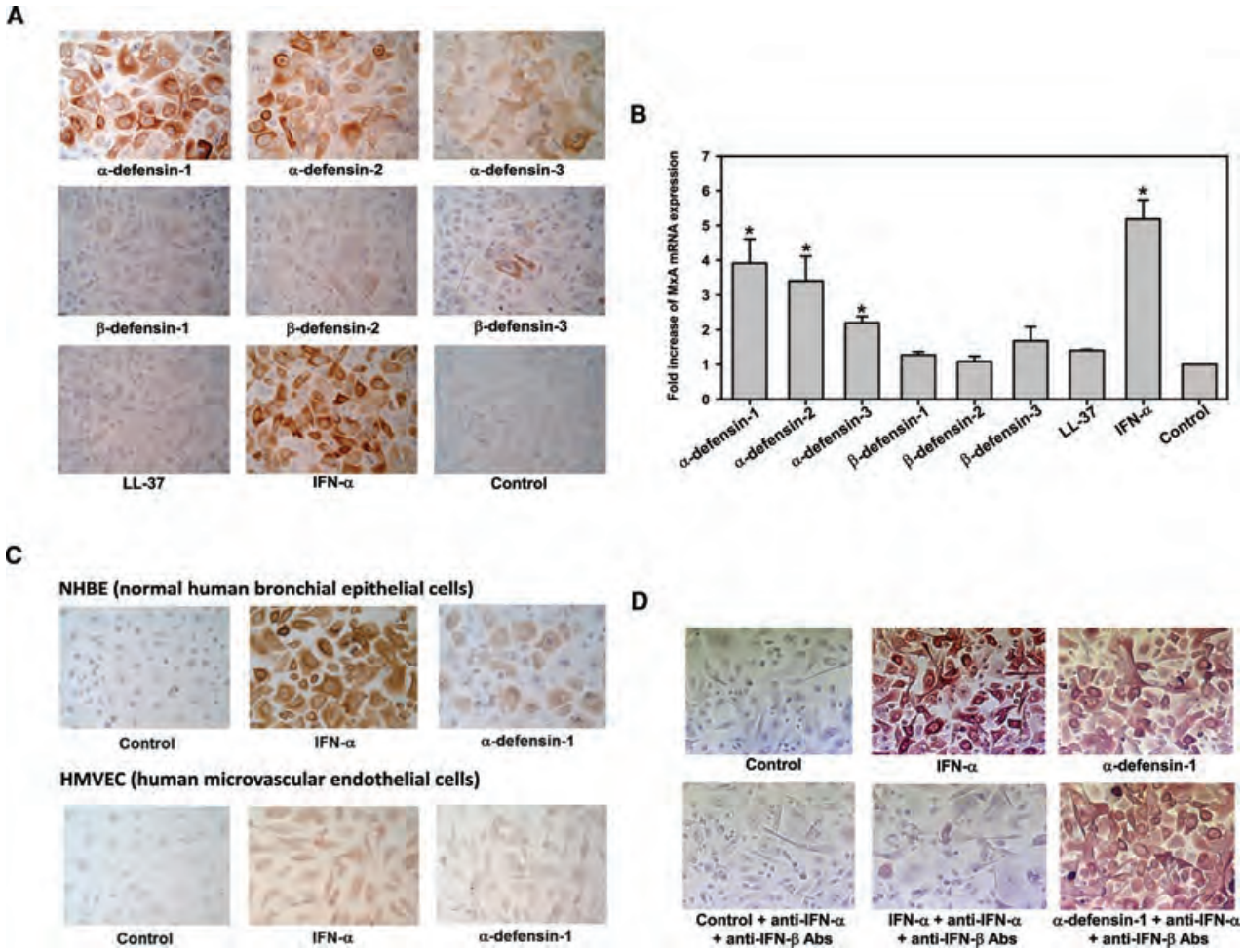

Figure 3. α-defensins-induced protein and mRNA expression of MxA in HGECs and other cells. (A) HGECs were treated for 24 h with the indicated antimicrobial peptides as described in the *Materials and methods*. IFN-α was used as a positive control and culture medium as a negative control. Immunohistochemical staining with anti-MxA antibody was used to detect MxA protein in treated HGECs. Data are representative of four separate experiments. One sample per experiment and each sample derived from different individual donors. (B) Real-time RT-PCR was also used to analyze MxA mRNA expression in 6 h treated HGECs. Data are demonstrated as the mean fold increase + SEM of MxA mRNA expression compared with medium control after normalization with the GAPDH of four separate experiments. One sample per experiment and each sample derived from different individual donors ($^*p < 0.05$, compared with medium control). (C) Normal human bronchial epithelial cells and primary human microvascular endothelial cells were treated for 24 h with α-defensin-1. Immunohistochemical staining with anti-MxA antibody was used to detect MxA protein in treated cells. IFN-α was used as positive control and culture medium was used as negative control. Data are representative of four separated experiments. (D) HGECs were treated for 24 h with α-defensin-1 or IFN-α in the absence or presence of neutralizing antibodies against type I IFN (IFN-α and IFN-β). Culture medium was used as negative control. Immunohistochemical staining with anti-MxA antibody was used to detect MxA protein in treated HGECs. Data are representative of four separate experiments. One sample per experiment and each sample derived from different individual donors.

PMN-derived-α-defensins induce MxA expression in HGECs

α-defensins are known as major proteins secreted by PMNs [32]. In the physiological condition of healthy gingiva, PMNs and their products are present in the tissue and the crevicular fluid in the gingival sulcus [33, 34].

In vitro culture of PMNs (5×10^6 cells/mL) for 6 h led to secretion of α-defensins in supernatants (which ranged from 90 479 to 98 714 pg/mL). To investigate the role of the PMN-derived α-defensins in MxA expression, we cultured HGECs with 6 h PMN supernatants. Under this condition, expression of MxA at both mRNA and protein levels in HGEC was observed after 6 h and 24 h treatment, respectively (Fig 5A and B). The MxA-inducing activity was diminished when neutralizing antibody against α-defensins was added to the culture, whereas neutralizing antibodies against type I IFN (IFN-α and IFN-β) had no effect (Fig. 5B). These data suggest that PMN-derived α-defensins were responsible for the observed MxA expression.

MxA and α-defensin protein expression in healthy gingival sulcus

The immunostaining results to detect epithelial MxA were obtained using the oral, but not the sulcus, side of periodontal tissue

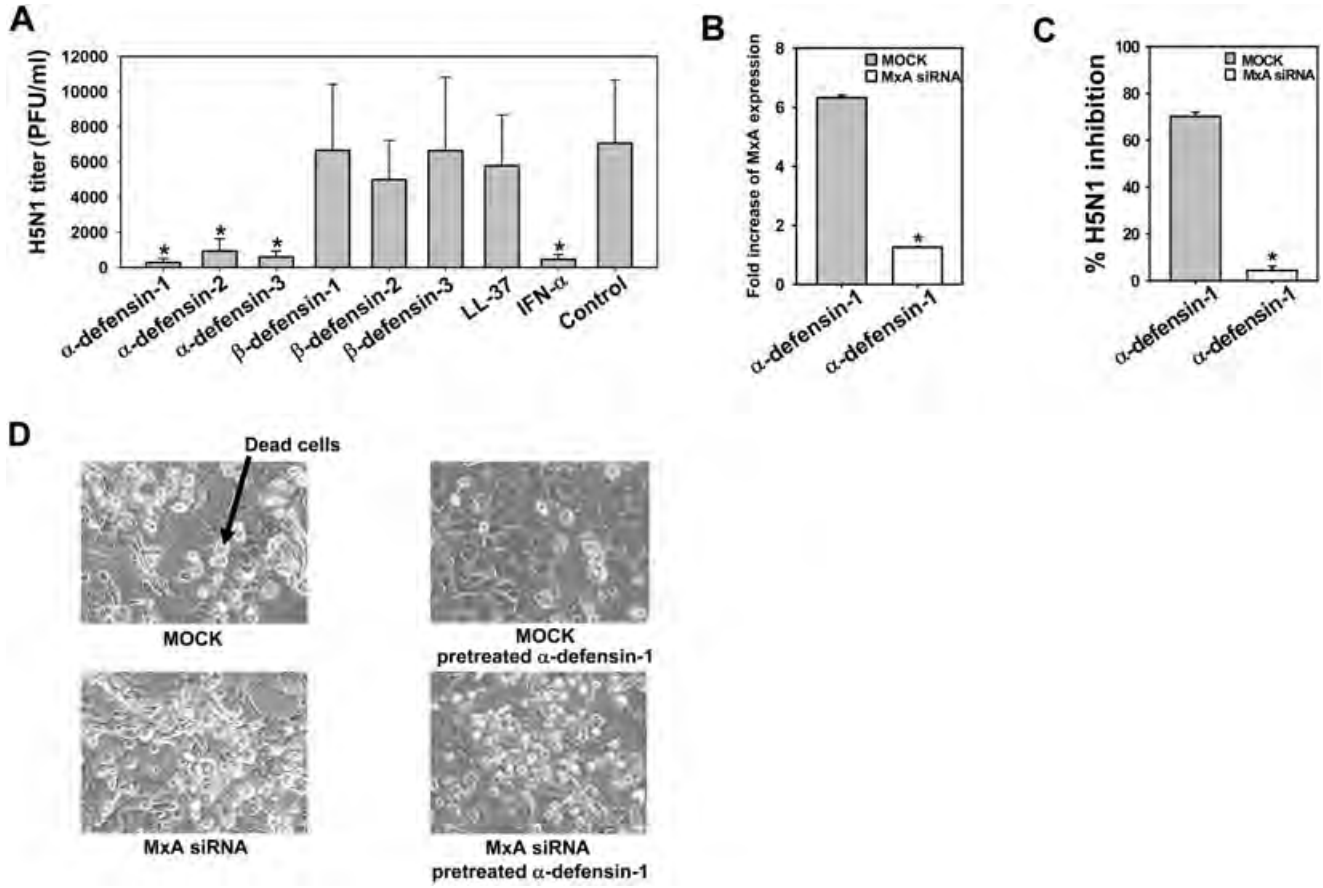

Figure 4. Protection against avian influenza H5N1 infection by MxA protein in α-defensin -pretreated HGECs. (A) HGECs pretreated for 24 h with either α-defensin-1; α-defensin-2; α-defensin-3; β-defensin-1; β-defensin-2; β-defensin-3; LL-37; IFN-α; or not pretreated were co-cultured with H5N1 virus at MOI 1. Viral titers in culture supernatant were determined at 48 h postinfection in HGECs as described in Materials and methods. Data are mean PFU/mL + SEM of four separate experiments. One sample per experiment and each sample derived from different individual donors ($^*p < 0.05$, compared with control). (B) HGECs were transfected with MxA siRNA or MOCK control and then treated with α-defensin-1. Real-time RT-PCR was used to analyze MxA mRNA expression in the cells. Data are demonstrated as the mean fold increase + SEM of MxA mRNA expression in α-defensin-1-treated HGECs that had been transfected with MxA siRNA or MOCK control, compared with medium control after normalization with the GAPDH of four separate experiments. One sample per experiment and each sample derived from different individual donors ($^*p < 0.05$, compared with MOCK control). (C) As in (B) but the transfected HGECs, which were treated with α-defensin-1, were then co-cultured with H5N1 virus at MOI 1 and inhibition of viral replication was subsequently measured. Data are mean percentage of H5N1 inhibition + SEM of four separate experiments. One sample per experiment and each sample derived from different individual donors ($^*p < 0.05$, compared with MOCK control). (D) As in (C) but treated cells were analyzed by light microscopy. Dead cells were rounded and differentiated from the polygonal-shaped healthy cells. Data are representative of four separate experiments. One sample per experiment and each sample derived from different individual donors.

(Fig. 2) because the epithelium at the sulcus side, especially for the junctional epithelium, is generally lost or torn during the surgical procedure. Fig. 6A depicts anatomic landmarks of the gingival sulcus. In this study, we were able to obtain two specimens of gingival sulcus area from healthy periodontal tissue. We then investigated localization of MxA protein in the healthy sulcus and also in relation to α-defensin. Fig. 6C shows that MxA protein was consistently expressed throughout epithelial cells of periodontal tissues. MxA staining was especially intense in the junctional epithelium (Fig. 6C). α-defensins were identified in small round cells with PMN morphology, most of which were found in the connective tissue layer (Fig. 6E). Migratory PMNs in junctional epithelium were also observed and highlighted in Fig. 6D. Thus, epithelial MxA protein was in close association with α-defensin-positive cells.

Discussion

To the best of our knowledge, the present study is the first to identify in humans the ability of α-defensins, endogenous antimicrobial peptides from PMNs, to induce the expression of epithelial MxA, a potent antiviral protein against both RNA and DNA viruses. This innate antiviral immune mechanism could play an important role in maintaining healthy periodontal tissues. α-defensin-induced MxA is an additional pathway to the well-recognized type I IFN

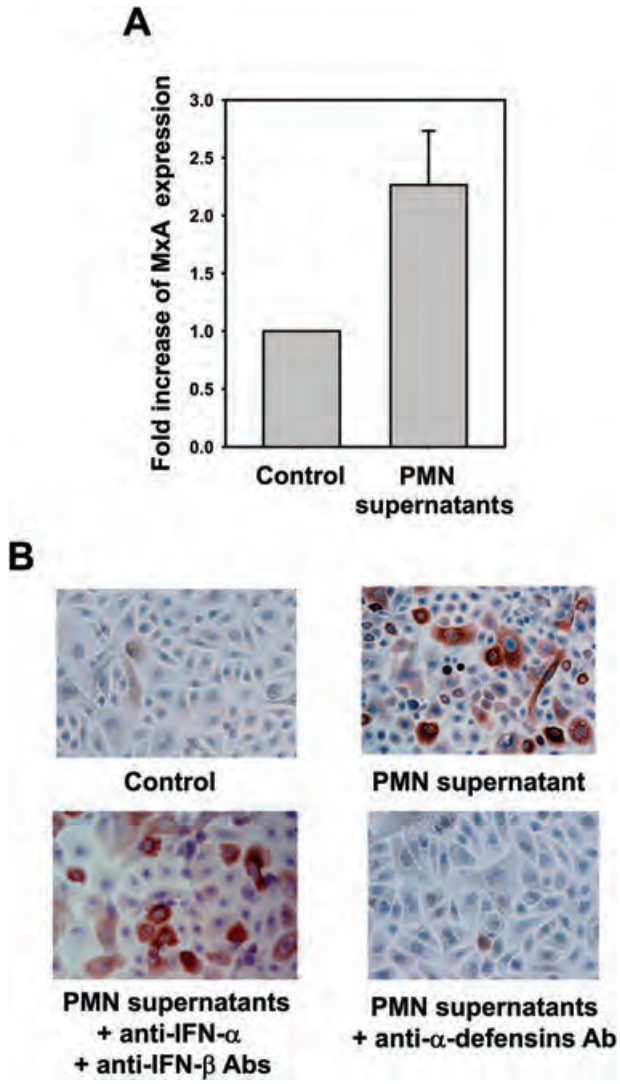

Figure 5. PMN-derived α-defensin-induced MxA expression in HGECs. (A) Real-time RT-PCR was used to analyze mRNA expression of MxA in HGEC cultures treated with PMN culture supernatants. Culture medium was used as negative control. Data are demonstrated as the mean fold increase + SEM of MxA mRNA expression after normalization with the GAPDH of four separate experiments. One sample per experiment and each sample derived from different individual donors. Control is set to 1. (B) Immunohistochemical staining with anti-MxA antibody was used to detect MxA in HGEC cultures treated with either: PMN culture supernatants; PMN culture supernatants plus neutralizing antibodies against type I IFN (IFN-α and IFN-β); or PMN culture supernatants plus neutralizing antibodies against α-defensins. Culture medium was used as negative control. Data are representative of four separate experiments. One sample per experiment and each sample derived from different individual donors.

induction [35, 36]. This function seems to be unique to α-defensin, because other antimicrobial peptides in healthy periodontal tissue (β-defensins and LL-37) induced only negligible MxA expression. It should be noted that α-defensins are known to upregulate co-stimulatory molecule and CD91 expression on antigen presenting dendritic cells [37].

There is little available information regarding innate antiviral immunity in the oral cavity. The human mouth harbors millions of microbes; however, we rarely develop serious infections [38]. Our previous research demonstrated TLRs and RLRs, key microbial sensors, in cells of periodontal tissues, which are critical for innate immune activation and local defense [7–9]. In the present study, we observed expression of MxA, PKR, OAS, and SLPI in healthy periodontal tissues, thus highlighting the role of innate antiviral immunity in periodontal tissue.

MxA proteins are key mediators of innate antiviral resistance induced in cells by type I (α/β) and type III (λ) IFNs [29]. The human *MxA* gene belongs to the class of IFN-stimulated genes (ISGs) and it is used as a surrogate marker for type I IFN activity in various experimental and clinical settings. Santoro et al. [39] used MxA to identify type I IFN in oral lichen planus. They found large numbers of MxA-positive cells in the lesion; therefore, a role of type I IFN in the pathology of oral lichen planus was postulated.

We are unaware of any previous study of MxA in periodontal disease. Our consistent finding of positive immunostaining of MxA protein in epithelium of healthy periodontal tissues (n = 9) was somewhat unexpected, since real-time PCR detected only negligible expression of type I IFN or type III IFN in healthy tissue specimens. Interestingly, the level of MxA proteins in the epithelial layer was significantly higher in healthy periodontal tissues than in periodontitis (Table 1). While searching for candidate MxA inducers, we treated primary HGECs with a variety of antimicrobial molecules, which are constitutively expressed in gingival epithelium. We clearly observed MxA protein expression after treatment with α-defensin-1, -2, or -3, but not with the other antimicrobial peptides β-defensin-1, -2, -3, or LL-37.

At present, it is not clear how α-defensins induce MxA expression. Our data strongly suggest that induction of MxA expression by α-defensin-1 is not dependent on type I IFN as neutralizing antibodies against type I IFN had no effect on the MxA expression. The mechanism of α-defensin-1-induced MxA expression is different from IFN-α-induced MxA expression since we could not detect the enhanced expression of other ISGs including STAT1, STAT2, IRF3, IRF7, and IRF9 (Supporting Information Fig. 2). Furthermore, STAT1 activation was also not detected in α-defensin-1-treated HGECs (Supporting Information Fig. 3). This observation is in line with previous study which showed that α-defensin-1 did not induce STAT1 activation in HeLa-CD4 cells [40]. The α-defensin-1-induced MxA expression was not specific to HGECs since this effect was also observed in normal human bronchial epithelial cells and primary human microvascular endothelial cells. These findings are supported by recent observations showing that human α-defensin-1 induced homologue MxA in fish cell line [31]. Our results may also explain the previous observation which demonstrated that MxA can be induced in lipopolysaccharide (LPS) stimulated PMNs independent of type I IFN [41]. It is possible that LPS stimulated PMNs to release α-defensins, resulting in MxA expression.

MxA is a protein with broad antiviral activity; it blocks viral replication at an early stage [42]. We demonstrated that MxA

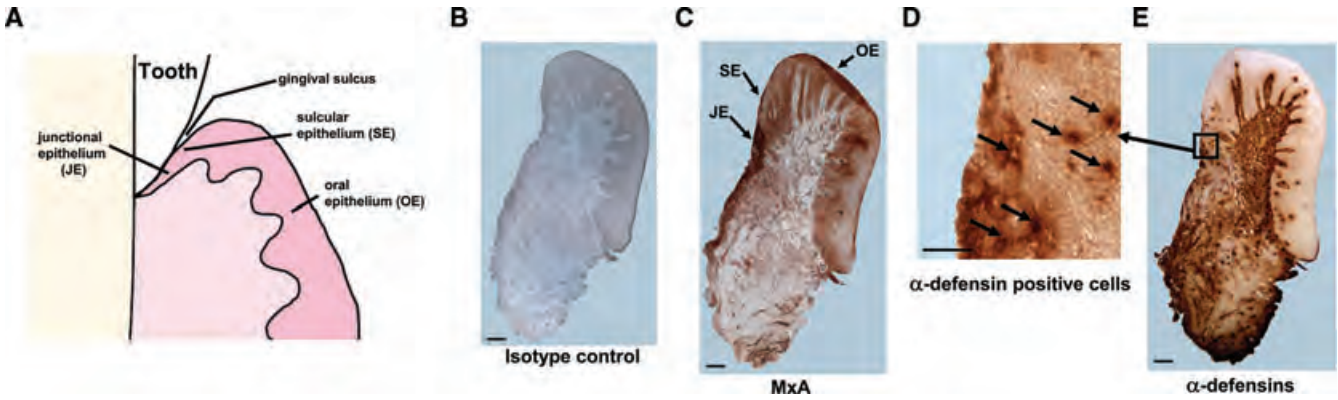

Figure 6. MxA and α-defensin protein expression in healthy gingival sulcus. (A) Diagram showing anatomic landmarks of the gingival sulcus area. Healthy periodontal tissue including the gingival sulcus area was stained with (B) isotype control, (C) anti-MxA antibody, and (D, E) anti-α-defensin antibody by immunoperoxidase technique and counterstained with hematoxylin. Data are representative of two different individual tissue specimens. Scale bars represent 200 μM in (B), (C), and (E), and 50 μM in (D).

expressed in α-defensin-treated HGECs inhibited avian influenza H5N1 viral replication. After silencing the *MxA* gene, HGECs treated with α-defensin-1 robustly downregulated MxA function, allowing viral replication and cell death to occur. It is tempting to speculate that MxA expression in periodontal tissue may have a role in antiviral defense during the consumption of H5N1-infected poultry meat; however, further research is required. It should be noted that α-defensins are known to directly inactivate viruses and inhibit their entry [43]. Our results provide additional antiviral pathway by which α-defensins modulate host cells to express MxA protein and inhibit viral replication.

PMNs are a major source of α-defensins. Our in vitro data demonstrated that when neutralizing antibody against α-defensins was added to the PMN supernatant-treated HGEC culture, the MxA-inducing activity was diminished. Therefore, α-defensins released from PMNs are likely to be responsible for the observed MxA expression in periodontal tissue. The intense MxA staining observed in the gingival sulcus area may be related to the pathway of a constant migration of PMNs from subepithelial connective tissue vessels through junctional epithelium and into this area [44]. This dynamic sequence suggests a crosstalk between resident nonimmune cells, the epithelium, and professional phagocytic cells, PMNs, all of which are essential for local innate immune activation.

It is interesting to note that MxA expression was lower in diseased periodontal tissue which commonly has more infiltrated PMNs as compared with healthy periodontal tissue. One could speculate that downregulation of the observed MxA expression in periodontitis could result from suppressor proteins produced during intense inflammatory reaction. The increased expression of suppressors of cytokine signaling (SOCS) proteins in periodontitis was recently reported [45]. Both SOCS-1 and SOCS-3 are able to inhibit MxA expression [46].

In conclusion, this study demonstrates that α-defensins, antimicrobial peptides constitutively expressed in healthy periodontal tissue, induce expression of a classical antiviral protein, MxA, in gingival epithelium. Strong MxA activity at the strategic gingival sulcus, in close proximity to microbial plaque, may serve as one of the important innate tools in maintaining periodontal homeostasis. We believe that our findings warrant further research into the physiological role of α-defensin-induced MxA in the antiviral response of the periodontal tissue.

Materials and methods

Reagents

Antimicrobial peptides: human α-defensin-1, -2, and -3, human β-defensin-1, -2, and -3, and LL-37 were obtained from Innovagen (Lund, Sweden). IFN-α and neutralizing antibodies against IFN-α and IFN-β were purchased from PBL Biomedical Laboratory (Piscataway, NJ, USA). Neutralizing antibody against α-defensins was obtained from Hycult biotech (Uden, The Netherlands). Polymorphprep was purchased from Axis-Shield PoC AS (Oslo, Norway).

Human periodontal tissue sample collection

Tissue specimens were collected from patients (one biopsy per one patient) at Periodontal Clinic and Department of Oral Maxillofacial Surgery, Faculty of Dentistry, Chulalongkorn University. The ethical approval by the ethics committee of Faculty of Dentistry, Chulalongkorn University and informed consent of all participating subjects were obtained before operation. Healthy periodontal tissue samples were collected from sites with clinically healthy gingiva (no gingival inflammation, probing depth < 4 mm, and no radiographic bone loss) during crown-lengthening procedure for prosthetic reasons. Severe periodontitis tissue samples were collected from sites of extracted teeth with hopeless prognosis (inflamed gingiva, probing depth > 6 mm, and bone loss $> 60\%$ of the root).

Periodontal tissue specimens used for immunostaining, real-time quantitative RT-PCR, and in vitro cultures were derived from different donors.

Human gingival epithelial cells (HGECs)

The primary HGECs, derived from healthy periodontal tissue, were obtained following established procedure [9]. In brief, the excised tissues were immediately washed with Dulbecco's phosphate buffered saline and digested in 0.2% dispase for 24 h at 4°C. The separated epithelial layer was washed, minced, and cultured in a serum-free keratinocyte growth medium (Clonetics, Walkersville, MD, USA) supplemented with human recombinant epidermal growth factor, hydrocortisone, bovine insulin, bovine pituitary extract, gentamicin sulfate, amphotericin B, and 0.15 mM $CaCl_2$. The HGEC cultures at passage two to four were used throughout the study.

Real-time RT-PCR

Total RNA from periodontal tissue samples and HGECs were isolated by using an RNeasy Mini kit from Qiagen (Hilden, Germany). One microgram of DNase I-treated total RNA were reverse transcribed using ImProm-II Reverse Transcription System for RT-PCR (Promega, Madison, WI, USA). The cDNA was then divided and used for PCR amplification of antiviral protein and cytokine expression. Real-time RT-PCR assays were performed on LightCycler System 480 (Roche Molecular Diagnostics, Mannhein, Germany) using SYBR Green PCR Master Mix (Roche Molecular Diagnostics). MxA, PKR, OAS, SLPI, IFN-α, IFN-β, IFN-λ, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were amplified using specific primers purchased from Operon (Ebersberg, Germany). The primer sequences are shown below. MxA (5′-GCTACACACCGTGACGGATATGG-3′/5′-CGAGCTGG ATTGGAAAGCCC-3′), PKR (5′-GCCTTTTCATC CAAATGG AATTC-3′/5′-GAAATC TGTTCTGGGCTCATG-3′), OAS (5′-CATCCGCCTAGTCAAGCACTG-3′/5′-CCACCACCCAAGTTT CC TGTAG-3′), SLPI (5′-TTCCCCTGTGAAAGCTTGATTC-3′/5′-GATA TCAGTGGTGGAGCCAAGTC-3′), IFN-α (5′-GGATGAGACC CTCCTAGACAAAT-3′/5′-ATGATTTCTGCTCTGACAACCTC-3′), IFN-β (5′-GATTCATCTAGCACTGGCTGG-3′/5′-CTTCAGGTA ATGCAGAATCC-3′), IFN-λ (5′-GGACGCCTTGGAAGAGTCACT-3′/5′-AGAAGCCTCAGGTCCCAATTC-3′), and GAPDH (5′-GA AGGCTGGGGCTCATTT-3′/5′-CAGGAGGCATTGCTGATGAT-3′).

Amplification conditions, sequences, and concentrations of the primers were similar to those of RT-PCR. After 45 reaction cycles, the melting curve analysis was performed at 95°C for 5 s, 65°C for 1 min, and heating to 97°C using a ramp rate of 0.11°C/sec with continuous monitoring of fluorescence. The melting peak generated represented the specific amplified product. All samples had only a single peak, indicating a pure product and no primer/dimer formation. Amplicons of a single band with the expected sizes were also confirmed in all reactions by agarose gel electrophoresis. The amplification efficiencies were high (close to 100%) when multiple standard curves were performed using serial mRNA dilutions.

For periodontal tissue specimens, the relative mRNA expression of antiviral proteins and cytokines was normalized to corresponding GAPDH for each sample, using the formula $= 2^{-\Delta CT}$, where $\Delta CT = C_{T\text{-}geneX}\text{-}C_{T\text{-}GAPDH}$. The relative quantification of mRNA expression in periodontitis tissues was presented as the mean fold increase ± SEM, using the mean value obtained from the healthy tissue as a reference (relative quantification = 1).

For HGEC culture, fold differences in mRNA expression levels of antiviral proteins and cytokines between sample A and sample B was calculated using the $\Delta\Delta C_T$ method [47]. Levels of gene of interest were normalized to corresponding GAPDH for each sample, and the fold increase between sample A and sample B was calculated as follows: Fold increase $= 2^{-\Delta\Delta CT,}$ where

$$-\Delta\Delta C_T = (C_{T\text{-}geneX} - C_{T\text{-}GAPDH})_{sampleA} - (C_{T\text{-}geneX} - C_{T\text{-}GAPDH})_{sampleB}$$

Immunohistochemical analysis of MxA protein and α-defensins in periodontal tissue

The excised periodontal tissues were immediately washed in normal saline solution, placed in the optimum cutting temperature embedding compound, snap-frozen in liquid nitrogen, and stored at −80°C. Single immunohistochemical staining was performed via Polymer/HRP and DAB+ chromagen system (DAKO EnVision™ G/2 Doublestain System, Glostrup, Denmark) on the frozen sections. After fixation in acetone, they were stained with primary mouse-anti-human mAb against human-MxA (clone M143, Dr. Haller, University of Freiburg, Freiburg, Germany), human α-defensins, or isotype control.

Three selective areas of oral epithelium: upper, middle, and lower parts of each tissue specimen were counted for MxA positive cells. The immunoreactivity of MxA staining was given a semiquantitative score ranging from score 1–3. Score 1 = the area of positive cells was less than 10% in the counting field, score 2 = 10–50%, and score 3 = more than 50%.

Effects of antimicrobial peptides on HGECs

Nontoxic concentrations of different antimicrobial peptides for HGECs were predetermined as assessed by cell viability (MTT assay and Trypan blue exclusion). HGECs, normal human bronchial epithelial cells (Clonetics) and primary human microvascular endothelial cells (Clonetics) were treated with nontoxic doses of either α-defensin-1 (10 μM); α-defensin-2 (10 μM); α-defensin-3 (10 μM); β-defensin-1 (10 μM); β-defensin-2 (10 μM); β-defensin-3 (0.5 μM); LL-37 (2 μg/mL); or IFN-α (100 U/mL). After 6 h of treatment with antimicrobial peptide or cytokine, mRNA expression of MxA was analyzed. In neutralization experiment, cells were treated with α-defensin-1 or IFN-α in the absence or presence of neutralizing antibodies against IFN-α (400 neutralization unit/mL) and IFN-β (400 neutralization unit/mL). After

24 h of treatment, immunohistochemical analysis of MxA protein was carried out.

Virus

H5N1 virus (A/open-billed stork/Nahkonsawan/BBD0104F/04) was isolated from cloacal swabs of live Asian open-billed storks between 2004–2005 and propagated in Madin-Darby canine kidney cells using MEM (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Hyclone, Logan, UT, USA) and penicillin and streptomycin [48]. The sequence data of the virus was submitted to GenBank with accession numbers DQ989958. The virus was grown in Madin-Darby canine kidney cells and the titer of virus stock was determined as described previously [48]. All experiments with H5N1 virus were performed in a Biosafety Level 3 facility (Mahidol University) by trained researchers.

H5N1-infected HGECs

HGECs (40,000 cells/well) were treated with either α-defensin-1 (10 μM); α-defensin-2 (10 μM); α-defensin-3 (10 μM); β-defensin-1 (10 μM); β-defensin-2 (10 μM); β-defensin-3 (0.5 μM); LL-37 (2 μg/mL); or IFN-α (100 U/mL) for 24 h. They were washed two times and then co-cultured with H5N1 virus at MOI 1 (1 PFU/cell). After 1 h, the inoculum virus was removed and the HGECs were washed two times with PBS and cultured with fresh medium. Virus titers in culture supernatants and cytopathic effect were determined 48 h postinfection. To assess the number of infectious particles (plaque titers) in cell culture supernatants, a plaque assay using Avicel (RC-591, FMC Biopolymer, Germany) was performed in 96-well plates [49, 50]. Virus-infected cells were immunostained by incubating for 1 h with a mAb specific for the influenza A virus nucleoprotein (Chemicon, Temecula, CA, USA) followed by 30-min incubation with peroxidase-labeled anti-mouse antibody (Southern Biotech, Birmingham, AL, USA) and 30-min incubation with True blue™ peroxidase substrate (KPL, Gaithersburg, MD, USA). The PFU were counted with ELISPOT reader (Ziess, Germany). Percentage of H5N1 inhibition was then calculated.

Cell death reflecting cytopathic effect of H5N1 infection was observed under a microscope. All experiments with H5N1 virus were performed in a Biosafety Level 3 facility.

siRNA, transfection, and infection

MxA siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Briefly, semi-confluent HGECs were seeded with growth media without antibiotics 1 day before transfection. 80 nM MxA siRNA and 5 μL siRNA tranfection reagent were diluted in 1 mL of transfection medium, mixed, and incubated at room temperature for 45 min. HGECs were washed two times with transfection medium and then the dilutes MxA siRNA was added for 7 h, then 2× growth medium was added and cells were cultured overnight. Depletion of MxA expression by MxA siRNA was assessed by real-time RT-PCR and immunohistochemistry (for protein level). Transfected HGECs were treated with α-defensin-1 overnight and then infected with H5N1 virus.

PMN culture

Highly purified PMNs from healthy human subjects were prepared by density centrifugation using Polymorphprep™. The purity of PMNs was > 95%, as determined by anti-CD16 mAb using flow cytometry. PMNs (5×10^6 cells/mL) were incubated for 6 h in serum-free keratinocyte growth medium. Supernatants were collected for measurement of α-defensin production by ELISA (detected all human α-defensin-1, -2, and -3). PMN supernatants with or without neutralizing antibody against α-defensins (neutralizes all human α-defensin-1, -2, and -3; 1 μg/mL) or neutralizing antibodies against IFN-α (400 neutralization unit/mL) and IFN-β (400 neutralization unit/mL) was added to HGEC cultures. After 6 h of treatment with either PMN supernatant or medium control, mRNA expression of MxA was analyzed by real-time RT-PCR. After 24 h incubation, MxA protein expression in HGECs was analyzed by immunohistochemistry.

Statistical analysis

The parametric Student's *t*-test was used for normally distributed data, and the nonparametric Mann–Whitney rank-sum test was used for nonnormally distributed data. A p-value < 0.05 was considered statistically significant. Data were analyzed with SPSS Version 11.5 software (SPSS Inc., Chicago, IL, USA).

Acknowledgements: This work was supported by BRG5380011 from Thailand Research Fund, Chulalongkorn University, and Ratchadapisek endowment. The authors thank S. Wiboon-ut (Department of Microbiology, Faculty of Science, Mahidol University) for technical assistance with avian influenza H5N1 experiments. We also thank Dr. C. Champaiboon for tissue sample collection, P. Ekchariyawat for STAT1 activation experiment, and Dr. K. Torrungruang for valuable comments and suggestions.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

1 **Slots, J.**, Herpesviral-bacterial interactions in periodontal diseases. *Periodontol. 2000* 2010. **52**: 117–140.

2 **Slots, J.**, Human viruses in periodontitis. *Periodontol. 2000* 2010. **53**: 89–110.

3 **Contreras, A. and Slots, J.**, Mammalian viruses in human periodontitis. *Oral Microbiol. Immunol.* 1996. **11**: 381–386.

4 **Contreras, A., Zadeh, H. H., Nowzari, H. and Slots, J.**, Herpes virus infection of inflammatory cells in human periodontitis. *Oral Microbiol. Immunol.* 1999. **14**: 206–212.

5 **Contreras, A., Nowzari, H. and Slots, J.**, Herpes viruses in periodontal pocket and gingival tissue specimens. *Oral Microbiol. Immunol.* 2000. **15**: 15–18.

6 **Parra, B. and Slots, J.**, Detection of human viruses in periodontal pockets using polymerase chain reaction. *Oral Microbiol. Immunol.* 1996. **11**: 289–293.

7 **Mahanonda, R.** and **Pichyangkul, S.**, Toll-like receptors and their role in periodontal health and disease. *Periodontol. 2000* 2007. **43**: 41–55.

8 **Mahanonda, R., Sa-Ard-Iam, N., Montreekachon, P., Pimkhaokham, A., Yongvanichit, K., Fukuda, M. M. and Pichyangkul, S.**, IL-8 and IDO expression by human gingival fibroblasts via TLRs. *J. Immunol.* 2007. **178**: 1151–1157.

9 **Mahanonda, R., Sa-Ard-Iam, N., Eksomtramate, M., Rerkyen, P., Phairat, B., Schaecher, K. E., Fukuda, M. M. et al.**, Cigarette smoke extract modulates human beta-defensin-2 and interleukin-8 expression in human gingival epithelial cells. *J. Periodontal Res.* 2009. **44**: 557–564.

10 **Beklen, A., Hukkanen, M., Richardson, R. and Konttinen, Y. T.**, Immunohistochemical localization of Toll-like receptors 1-10 in periodontitis. *Oral Microbiol. Immunol.* 2008. **23**: 425–431.

11 **Kubota, K., Sakaki, H., Imaizumi, T., Nakagawa, H., Kusumi, A., Kobayashi, W., Satoh, K. et al.**, Retinoic acid-inducible gene-I is induced in gingival fibroblasts by lipopolysaccharide or poly IC: possible roles in interleukin-1beta, -6 and -8 expression. *Oral Microbiol. Immunol.* 2006. **21**: 399–406.

12 **Sakaki, H., Imaizumi, T., Matsumiya, T., Kusumi, A., Nakagawa, H., Kubota, K., Nishi, N. et al.**, Retinoic acid-inducible gene-I is induced by interleukin-1beta in cultured human gingival fibroblasts. *Oral Microbiol. Immunol.* 2005. **20**: 47–50.

13 **Kawai, T. and Akira, S.**, The roles of TLRs, RLRs and NLRs in pathogen recognition. *Int. Immunol.* 2009. **21**: 317–337.

14 **Haller, O., Staeheli, P. and Kochs, G.**, Interferon-induced Mx proteins in antiviral host defense. *Biochimie* 2007. **89**: 812–818.

15 **Honda, K., Takaoka, A. and Taniguchi, T.**, Type I interferon [corrected] gene induction by the interferon regulatory factor family of transcription factors. *Immunity* 2006. **25**: 349–360.

16 **Theofilopoulos, A. N., Baccala, R., Beutler, B. and Kono, D. H.**, Type I interferons (alpha/beta) in immunity and autoimmunity. *Annu. Rev. Immunol.* 2005. **23**: 307–336.

17 **Goebel, C., Mackay, L. G., Vickers, E. R. and Mather, L. E.**, Determination of defensin HNP-1, HNP-2, and HNP-3 in human saliva by using LC/MS. *Peptides* 2000. **21**: 757–765.

18 **Mathews, M., Jia, H. P., Guthmiller, J. M., Losh, G., Graham, S., Johnson, G. K., Tack, B. F. et al.**, Production of beta-defensin antimicrobial peptides by the oral mucosa and salivary glands. *Infect. Immun.* 1999. **67**: 2740–2745.

19 **Murakami, M., Ohtake, T., Dorschner, R. A. and Gallo, R. L.**, Cathelicidin antimicrobial peptides are expressed in salivary glands and saliva. *J. Dent. Res.* 2002. **81**: 845–850.

20 **Crombie, R., Silverstein, R. L., MacLow, C., Pearce, S. F., Nachman, R. L. and Laurence, J.**, Identification of a CD36-related thrombospondin 1-binding domain in HIV-1 envelope glycoprotein gp120: relationship to HIV-1-specific inhibitory factors in human saliva. *J. Exp. Med.* 1998. **187**: 25–35.

21 **McNeely, T. B., Dealy, M., Dripps, D. J., Orenstein, J. M., Eisenberg, S. P. and Wahl, S. M.**, Secretory leukocyte protease inhibitor: a human saliva protein exhibiting anti-human immunodeficiency virus 1 activity in vitro. *J. Clin. Invest.* 1995. **96**: 456–464.

22 **White, M. R., Helmerhorst, E. J., Ligtenberg, A., Karpel, M., Tecle, T., Siqueira, W. L., Oppenheim, F. G. et al.**, Multiple components contribute to ability of saliva to inhibit influenza viruses. *Oral Microbiol. Immunol.* 2009. **24**: 18–24.

23 **Haller, O. and Kochs, G.**, Interferon-induced mx proteins: dynamin-like GTPases with antiviral activity. *Traffic* 2002. **3**: 710–717.

24 **Netherton, C. L., Simpson, J., Haller, O., Wileman, T. E., Takamatsu, H. H., Monaghan, P. and Taylor, G.**, Inhibition of a large double-stranded DNA virus by MxA protein. *J. Virol* 2009. **83**: 2310–2320.

25 **Pavlovic, J., Haller, O. and Staeheli, P.**, Human and mouse Mx proteins inhibit different steps of the influenza virus multiplication cycle. *J. Virol.* 1992. **66**: 2564–2569.

26 **Jana, N. K., Gray, L. R. and Shugars, D. C.**, Human immunodeficiency virus type 1 stimulates the expression and production of secretory leukocyte protease inhibitor (SLPI) in oral epithelial cells: a role for in innate mucosal immunity. *J. Virol.* 2005. **79**: 6432–6440.

27 **Haller, O., Frese, M. and Kochs, G.**, Mx proteins: mediators of innate resistance to RNA viruses. *Rev. Sci. Tech.* 1998. **17**: 220–230.

28 **Brand, S., Beigel, F., Olszak, T., Zitzmann, K., Eichhorst, S. T., Otte, J. M., Diebold, J. et al.**, IL-28A and IL-29 mediate antiproliferative and antiviral signals in intestinal epithelial cells and murine CMV infection increases colonic IL-28A expression. *Am. J. Physiol. Gastrointest. Liver Physiol.* 2005. **289**: G960–G968.

29 **Holzinger, D., Jorns, C., Stertz, S., Boisson-Dupuis, S., Thimme, R., Weidmann, M., Casanova, J. L. et al.**, Induction of MxA gene expression by influenza A virus requires type I or type III interferon signaling. *J. Virol.* 2007. **81**: 7776–7785.

30 **Diamond, G., Beckloff, N. and Ryan, L. K.**, Host defense peptides in the oral cavity and the lung: similarities and differences. *J. Dent. Res.* 2008. **87**: 915–927.

31 **Falco, A., Mas, V., Tafalla, C., Perez, L., Coll, J. M. and Estepa, A.**, Dual antiviral activity of human alpha-defensin-1 against viral haemorrhagic septicaemia rhabdovirus (VHSV): inactivation of virus particles and induction of a type I interferon-related response. *Antiviral Res.* 2007. **76**: 111–123.

32 **Ganz, T.**, Defensins: antimicrobial peptides of innate immunity. *Nat. Rev. Immunol.* 2003. **3**: 710–720.

33 **Lundy, F. T., Orr, D. F., Shaw, C., Lamey, P. J. and Linden, G. J.**, Detection of individual human neutrophil alpha-defensins (human neutrophil peptides 1, 2 and 3) in unfractionated gingival crevicular fluid–a MALDI-MS approach. *Mol. Immunol.* 2005. **42**: 575–579.

34 **Dale, B. A., Kimball, J. R., Krisanaprakornkit, S., Roberts, F., Robinovitch, M., O'Neal, R., Valore, E. V. et al.**, Localized antimicrobial peptide expression in human gingiva. *J. Periodontal Res.* 2001. **36**: 285–294.

35 **von Wussow, P., Jakschies, D., Hochkeppel, H. K., Fibich, C., Penner, L. and Deicher, H.**, The human intracellular Mx-homologous protein is specifically induced by type I interferons. *Eur. J. Immunol.* 1990. **20**: 2015–2019.

36 **Ronni, T., Melen, K., Malygin, A. and Julkunen, I.**, Control of IFN-inducible MxA gene expression in human cells. *J. Immunol.* 1993. **150**: 1715–1726.

37 **Presicce, P., Giannelli, S., Taddeo, A., Villa, M. L. and Della Bella, S.**, Human defensins activate monocyte-derived dendritic cells, promote the production of proinflammatory cytokines, and up-regulate the surface expression of CD91. *J. Leukocyte Biol.* 2009. **86**: 941–948.

38 **Paster, B. J., Boches, S. K., Galvin, J. L., Ericson, R. E., Lau, C. N., Levanos, V. A., Sahasrabudhe, A. et al.**, Bacterial diversity in human subgingival plaque. *J. Bacteriol.* 2001. **183**: 3770–3783.

39 **Santoro, A., Majorana, A., Roversi, L., Gentili, F., Marrelli, S., Vermi, W., Bardellini, E. et al.**, Recruitment of dendritic cells in oral lichen planus. *J. Pathol.* 2005. **205**: 426–434.

40 **Chang, T. L., Francois, F., Mosoian, A. and Klotman, M. E.**, CAF-mediated human immunodeficiency virus (HIV) type 1 transcriptional inhibition is distinct from alpha-defensin-1 HIV inhibition. *J. Virol.* 2003. **77**: 6777–6784.

41 **Malcolm, K. C. and Worthen, G. S.**, Lipopolysaccharide stimulates p38-dependent induction of antiviral genes in neutrophils independently of paracrine factors. *J. Biol. Chem.* 2003. **278**: 15693–15701.

42 **Haller, O., Staeheli, P. and Kochs, G.**, Protective role of interferon-induced Mx GTPases against influenza viruses. *Rev. Sci. Tech.* 2009. **28**: 219–231.

43 **Daher, K. A., Selsted, M. E. and Lehrer, R. I.**, Direct inactivation of viruses by human granulocyte defensins. *J. Virol.* 1986. **60**: 1068–1074.

44 **Bosshardt, D. D. and Lang, N. P.**, The junctional epithelium: from health to disease. *J. Dent. Res.* 2005. **84**: 9–20.

45 **Garlet, G. P., Cardoso, C. R., Campanelli, A. P., Martins, W., Jr. and Silva, J. S.**, Expression of suppressors of cytokine signaling in diseased periodontal tissues: a stop signal for disease progression? *J. Periodontal Res.* 2006. **41**: 580–584.

46 **Vlotides, G., Sorensen, A. S., Kopp, F., Zitzmann, K., Cengic, N., Brand, S., Zachoval, R. et al.**, SOCS-1 and SOCS-3 inhibit IFN-alpha-induced expression of the antiviral proteins 2,5-OAS and MxA. *Biochem. Biophys. Res. Commun.* 2004. **320**: 1007–1014.

47 **Livak, K. J. and Schmittgen, T. D.**, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001. **25**: 402–408.

48 **Thitithanyanont, A., Engering, A., Ekchariyawat, P., Wiboon-ut, S., Limsalakpetch, A., Yongvanitchit, K., Kum-Arb, U. et al.**, High susceptibility of human dendritic cells to avian influenza H5N1 virus infection and protection by IFN-alpha and TLR ligands. *J. Immunol.* 2007. **179**: 5220–5227.

49 **Matrosovich, M., Matrosovich, T., Garten, W. and Klenk, H. D.**, New low-viscosity overlay medium for viral plaque assays. *J. Virol.* 2006. **3**: 1–7.

50 **Rowe, T., Abernathy, R. A., Hu-Primmer, J., Thompson, W. W., Lu, X., Lim, W., Fukuda, K. et al.**, Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. *J. Clin. Microbiol.* 1999. **37**: 937–943.

Abbreviations: **HGEC**: human gingival epithelial cell · **ISG**: IFN-stimulated gene · **MxA**: myxovirus resistance A · **OAS**: oligoadenylate synthetase · **PKR**: protein kinase R · **RLR**: retinoic acid-inducible gene-I-like receptor · **SLPI**: secretory leukocyte protease inhibitor · **SOCS**: suppressors of cytokine signaling

Full correspondence: Dr. Rangsini Mahanonda, Department of Periodontology, Faculty of Dentistry, Chulalongkorn University, Henry Dunant Rd., Bangkok 10330, Thailand
Fax: +662-2188861
e-mail: rangsini.m@chula.ac.th

Received: 11/4/2011
Revised: 26/10/2011
Accepted: 22/12/2011
Accepted article online: 4/1/2012

Reprint form Biochemical and Biophysical Research Communications

Biochemical and Biophysical Research Communications 398 (2010) 752–758

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Antiviral immune responses in H5N1-infected human lung tissue and possible mechanisms underlying the hyperproduction of interferon-inducible protein IP-10

Arunee Thitithanyanont [a], Anneke Engering [b], Monkol Uiprasertkul [c], Peeraya Ekchariyawat [a], Suwimon Wiboon-ut [a], Romchat Kraivong [a], Amporn Limsalakpetch [b], Utaiwan Kum-Arb [b], Kosol Yongvanitchit [b], Noppadol Sa-Ard-Iam [d], Pimprapa Rukyen [d], Rangsini Mahanonda [d], Kamon Kawkitinarong [e], Prasert Auewarakul [c], Pongsak Utaisincharoen [a], Stitaya Sirisinha [a], Carl J. Mason [b], Mark M. Fukuda [b], Sathit Pichyangkul [b,*]

[a] Faculty of Science, Mahidol University, Bangkok, Thailand
[b] U.S. Army Medical Component of the Armed Forces Research Institute of Medical Science, Bangkok, Thailand
[c] Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand
[d] Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand
[e] Chulalongkorn Medical School, Bangkok, Thailand

ARTICLE INFO

Article history:
Received 7 July 2010
Available online 11 July 2010

Keywords:
H5N1 autopsy
H5N1-infected human pulmonary cells
MxA
TNF-α
IFN-α
IP-10

ABSTRACT

Information on the immune response against H5N1 within the lung is lacking. Here we describe the sustained antiviral immune responses, as indicated by the expression of MxA protein and IFN-α mRNA, in autopsy lung tissue from an H5N1-infected patient. H5N1 infection of primary bronchial/tracheal epithelial cells and lung microvascular endothelial cells induced IP-10, and also up-regulated the retinoic acid-inducible gene-I (RIG-I). Down-regulation of RIG-I gene expression decreased IP-10 response. Co-culturing of H5N1-infected pulmonary cells with TNF-α led to synergistically enhanced production of IP-10. In the absence of viral infection, TNF-α and IFN-α also synergistically enhanced IP-10 response. Methylprednisolone showed only a partial inhibitory effect on this chemokine response. Our findings strongly suggest that both the H5N1 virus and the locally produced antiviral cytokines; IFN-α and TNF-α may have an important role in inducing IP-10 hyperresponse, leading to inflammatory damage in infected lung.

Published by Elsevier Inc.

1. Introduction

Infection with highly pathogenic avian influenza H5N1 virus, unlike most human influenza infection, causes severe disease with a case-fatality rate of about 60%. *In vitro* infection of human alveolar and bronchial epithelial cells with H5N1 viruses led to higher levels production of IFN-β, IL-6, RANTES, and especially IP-10 than in cells infected with human influenza H1N1 virus [1]. We recently demonstrated that human plasmacytoid dendritic cells (PDCs) produced high levels of IFN-α and TNF-α after exposure to H5N1 viruses [2]. Several studies have consistently described elevated blood levels of IP-10 and other cytokine/chemokine in H5N1 patients [3–5]. The increase in IP-10, MCP-1, MIG, and IL-8 plasma levels was significantly associated with fatality [3].

These findings provide an important link between serum cytokine/chemokine levels and clinical severity of H5N1 infection. However, they do not provide detailed information concerning immunopathology in the lung, the primary target organ of H5N1 infection. Due to a lack of histological specimens from infected patients, it has been difficult to systemically investigate the immune response against H5N1 in the lung, and to evaluate the contribution of this response to the pathogenesis of H5N1 infection. In an attempt to determine the pathological mechanism within infected lung tissue, we examined the antiviral immune response in autopsy lung tissue of a patient who died with H5N1 infection. We also investigated the possible mechanisms underlying the hyperproduction of IP-10 in H5N1-infected human lung.

2. Materials and methods

2.1. Virus

H5N1 virus (A/open-billed stork/Nahkonsawan/BBD0104F/04) was isolated from cloacal swabs of live Asian open-billed storks and propagated in Madin-Darby canine kidney cells [2].

* Corresponding author. Address: USAMC-AFRIMS, 315/6 Rajvithi Road, Bangkok 10400, Thailand. Fax: +66 2 644 4784.
E-mail address: sathitp@afrims.org (S. Pichyangkul).

0006-291X/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.bbrc.2010.07.017

2.2. Cell culture and viral infection

Human primary bronchial/tracheal epithelial cells and human microvascular endothelial cells (Cambrex) were cultured in BEBM and EBM-2 growth media, respectively. Cells of passage 3–4 (5×10^4 cells/well) were co-cultured with H5N1 virus at MOI 1 in the absence or presence of IFN-α and/or TNF-α. After 24 h of incubation, culture supernatants were collected and assessed for production of IP-10, IL-8 and IL-6. Influenza infection was confirmed by staining with FITC-conjugated anti-NP and M antibodies [2]. Peripheral blood mononuclear cells (PBMCs) from healthy donors were obtained by centrifugation using Histopaque (Sigma–Aldrich) and cultured (4×10^5 cells/well) in RPMI 1640 supplemented with nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μg/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen Life Technologie) containing 10% FCS.

In some experiments, primary human pulmonary cells were infected with H5N1 (MOI 1) in the presence TNF-α (6 ng/ml) and methylprednisolone (100 μg/ml) or atorvastatin (0.25–2 μM). IP-10 response was measured at 24 h after infection. Preliminary experiments were conducted to determine non-toxic concentrations of methylprednisolone and atorvastatin.

Recombinant human IFN-α β2 and recombinant human TNF-α were from PBL Biomedical Laboratories and R&D Systems, respectively. Methylprednisolone and atorvastatin were obtained from Pfizer. *Escherichia coli* LPS was purchased from InvivoGen.

2.3. Human tissue samples

Autopsy lung specimens from a H5N1 confirmed case and from a noninfectious patient were obtained from the archives of the Siriraj Hospital, Mahidol University. This investigation was approved by the Siriraj Ethics Committee, Mahidol University. The H5N1-infected patient was a 6-year-old boy who had progressive viral pneumonia leading to acute respiratory distress syndrome. He died on day 17 after onset of illness [6]. Autopsy lung tissue from one patient with no known respiratory infection was used as a negative control.

2.4. Real-time PCR

RNA was extracted from lung tissues as previously described [6]. cDNA was synthesized with AMV-RT (Promega, USA) using oligo-dT primer and then amplified by real-time PCR (Rotor Gene 3000, Corbett Research) with SYBR green I detection system. The sequences of IFN-α and IP-10 primers were as follows. IFN-α forward, 5′-AGA ATC ACT CTC TAT CTG AAA GAG AAG AAA TA-3′: IFN-α reverse, 5′-TCA TGA TTT CTG CTC TGA CAA CCT-3′; IP-10 forward, 5′-TCG AAG GCC ATC AAG AAT TT-3′; IP-10 reverse, 5′-GCT CCC CTC TGG TTT TAA GG-3′. Primers specific for the housekeeping genes; β-actin and GAPDH were as follows, β-actin forward, 5′-CCA CAC TGT GCC CAT CG-3′; β-actin reverse, 5′-AGG ATC TTC ATG AGG TAG TCA GTC AG-3′; GAPDH forward, 5′-GAT CAT CAG CAA TGC CTC CT-3′; GAPDH reverse, 5′-TGT GGT CAT GAG TCC TTC CA-3′. To assess RIG-I expression in human primary pulmonary cells, RNA of cells after 4 h of H5N1 infection was extracted with QIAGEN RNA easy kit (QIAGEN). cDNA was synthesized and amplified by real-time PCR with RIG-I forward primer, 5′-CTC TGC AGA AAG TGA AAG C-3′ and reverse primer, 5′-GGC TTG GGA TGT GGT CTA CT-3′. Copy number of each gene of interest and housekeeping gene in each sample were calculated by Rotor gene software using standard curve of known copy plasmid containing its own specific gene sequence. The relative expression of each gene of interest/housekeeping gene of the same sample was presented. The fold difference in gene expression was compared to the control sample.

2.5. siRNA, transfection, and infection

Stealth siControl (UAA GUG GUU GAC UUG AAC CUA AUG G) and Stealth siRIG-I (UAA GGU UGU UCA CAA GAA UCU GUG G) were purchased from Invitrogen. Transfection with siRNA was performed using lipofectamine 2000. Briefly, semi-confluent cells were seeded with growth media without antibiotics one day before transfection. Cells were transfected with siRNA in the Opti-MEM media for 4 h, then replaced with growth media, and cultured overnight. Depletion of RIG-I expression by siRNA RIG-I was assessed by real-time PCR. Transfected cells which had their RIG-I expression been silence were co-cultured with H5N1 virus (MOI 1), culture supernatants were collected at 24 h post infection and then measured for IP-10 production.

2.6. Immunohistochemistry

Tissue sections were prepared from archived formalin-fixed, paraffin-embedded lung tissues and were stained by immunohistochemistry. Primary antibody specific to MxA (Dr. Haller, University of Freiburg) was used. Detection of primary antibody was conducted by using Polymer/HRP and DAB + chromagen (EnVision-TM G/2 Doublestain System, Rabbit/Mouse, Dako).

2.7. Measurement of cytokines

Production of cytokines was measured by ELISA (R&D Systems).

2.8. Statistical analysis

Statistical comparisons among different treatment conditions with respect to production of IP-10 and TNF-α were conducted using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL). The parametric Student's *t* test was used for normally distributed data, and the non-parametric Mann–Whitney rank-sum test was used for non-normally distributed data. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Expression of MxA, IFN-α, and IP-10 in H5N1-infected human lung

The type I interferon-induced Mx proteins play a critical role in protection against influenza A viral infection [7]. However, their expression in H5N1-infected human lung has not been investigated. We examined the expression of human MxA in lung tissue from a six-year-old boy who died on day 17 after onset of H5N1 illness. The histopathological findings, sites of viral replication, and clinical data from this patient have been previously reported [6]. Immunohistochemistry data in Fig. 1A revealed the expression of cytoplasmic MxA protein in several different cell types in the patient's lung. Expression was especially strong in mucous gland epithelium, and also occurred in vascular endothelial and smooth muscle cells as well as alveolar epithelial cells. MxA expression was negligible in control autopsy lung tissue. The observed MxA expression in the H5N1-infected patient led us to hypothesize that this expression may reflect local induction of type I IFN. Consistent with this, we found increased expression of IFN-α mRNA (>700-fold increase) in H5N1-infected lung as compared to control lung (Fig. 1B). In addition to IFN-α, we detected increased expression of interferon-inducible protein IP-10 mRNA in H5N1-infected lung (>70-fold increase) as compared to control lung (Fig. 1C).

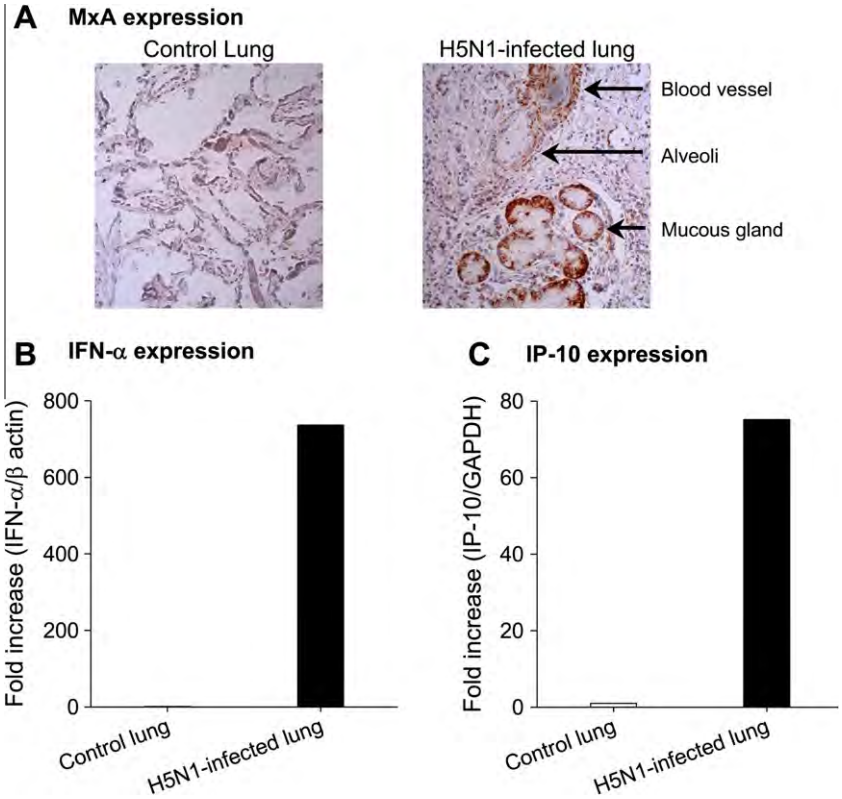

Fig. 1. Expression of MxA (A), IFN-α (B), and IP-10 (C) in autopsy lung tissue from human subjects with and without influenza H5N1 infection.

3.2. H5N1 infection stimulated IP-10 response which was mediated by RIG-I

The finding of enhanced expression of IP-10 in H5N1-infected lung prompted us to evaluate *in vitro* IP-10 response in H5N1-infected primary human bronchial/tracheal epithelial and lung microvascular endothelial cells. Co-culture of these cells with H5N1 (MOI 1) resulted in infection, demonstrated by immunofluorescence staining of intracellular viral proteins (Fig. 2A and B). Infectious viruses were also detected at 24 h postinfection in culture supernatants of both cell types (data not shown). Analysis of supernatants at 24 h after infection revealed production of IP-10, but only negligible IL-8 and IL-6 production (Fig. 2C and D).

RIG-I is critical for influenza A viral sensing and is involved in IP-10 response [8]. To better understand the role of RIG-I in H5N1-infected pulmonary cells, we analyzed the effect of H5N1 infection on this viral recognition receptor. Both cell types constitutively expressed low levels of RIG-I. However, infection by H5N1 for 4 h increased RIG-I mRNA expression in bronchial/tracheal epithelial cells 7-fold, and in lung microvascular endothelial cells 3-fold, as compared to non-infected cells (Fig. 2E).

To explore the role of RIG-I in H5N1-mediated IP-10 production, we transfected cells with RIG-I-targeted siRNA or control siRNA, and then infected them with H5N1 virus. Compared to transfection with control siRNA, RIG-I-targeted siRNA with 90% gene knockdown (Fig. 2F) induced reduction in IP-10 expression of 67% and 49% in H5N1-infected bronchial/tracheal epithelium and lung microvascular endothelium, respectively (Fig. 2G and H). Control lung cells and control siRNA-treated lung cells produced similar levels of IP-10 upon H5N1 infection (data not shown).

3.3. The influence of TNF-α and /or IFN-α on IP-10 response of H5N1-infected human pulmonary cells

Expression of TNF-α in the lung of this patient has been reported previously [6]. To mimic the microenvironment found in the lungs of infected patients, primary human pulmonary cells were co-cultured with H5N1 and cytokines expressed in H5N1-infected lung, i.e. IFN-α and TNF-α. Both cytokines are known to have anti-H5N1 activity [2]. After 24 h, culture supernatants were measured for IP-10 production. TNF-α (6 ng/ml) alone poorly induced IP-10 response (Fig. 3A and B). Combining TNF-α and H5N1 infection synergistically enhanced IP-10 production in both bronchial/tracheal epithelial ($P < 0.05$) and lung microvascular endothelial cells ($P < 0.05$) (Fig. 3A and B). That is, the effect of combined TNF-α treatment and H5N1 infection was statistically significantly greater than the sum of the individual effects. Live H5N1 virus was required to produce enhanced IP-10 production, as heat-killed virus did not increase production (data not shown). IFN-α (600 U/ml) alone induced a modest IP-10 response (Fig. 3A and B) in bronchial/tracheal epithelial cells, but not in lung microvascular endothelium. Treatment with IFN-α and H5N1 failed to enhance IP-10 response in both cell types (Fig. 3A and B).

A further increase in IP-10 response was observed in both types of pulmonary cells when adding IFN-α to the TNF-α and H5N1 treated cells ($P < 0.01$) (Fig. 3A and B). The increased response could result from the cooperative effect between IFN-α and TNF-α. To test this hypothesis, we co-cultured pulmonary cells in the presence of IFN-α and/or TNF-α in the absence of H5N1 infection. The combination of TNF-α (6 ng/ml) and IFN-α (600, 300 U/ml) synergistically induced high levels of IP-10 from both cell types ($P < 0.01$) (Fig. 3C and D).

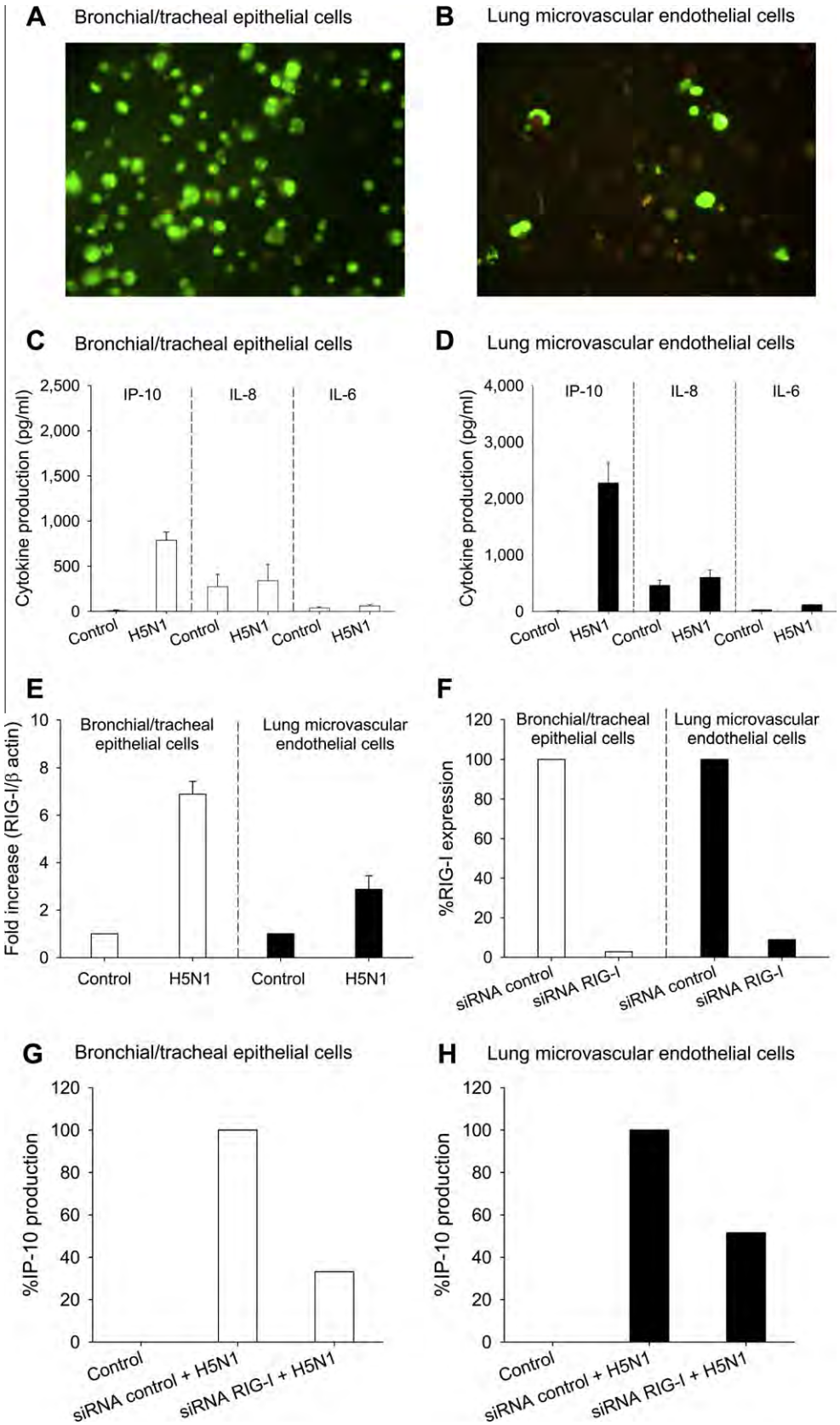

Fig. 2. *In vitro* infectivity by influenza H5N1 virus and induction of IP-10, IL-8, and IL-6 in bronchial/tracheal epithelial cells (A and C) and lung microvascular endothelial cells (B and D). H5N1 up-regulated RIG-I expression in bronchial/tracheal epithelial cells and lung microvascular endothelial cells (E). RIG-I expression (%) in epithelial and endothelial cells transfected with control siRNA or siRNA RIG-I after overnight culture (F). IP-10 expression (%) in H5N1 infected epithelial cells (G) and endothelial cells (H), transfected with control siRNA or siRNA RIG-I. Data in (F–H) are representative results from one of two experiments. Data in (C–E) are shown as mean values ± SEM of four independent experiments using cells derived from two individual donors.

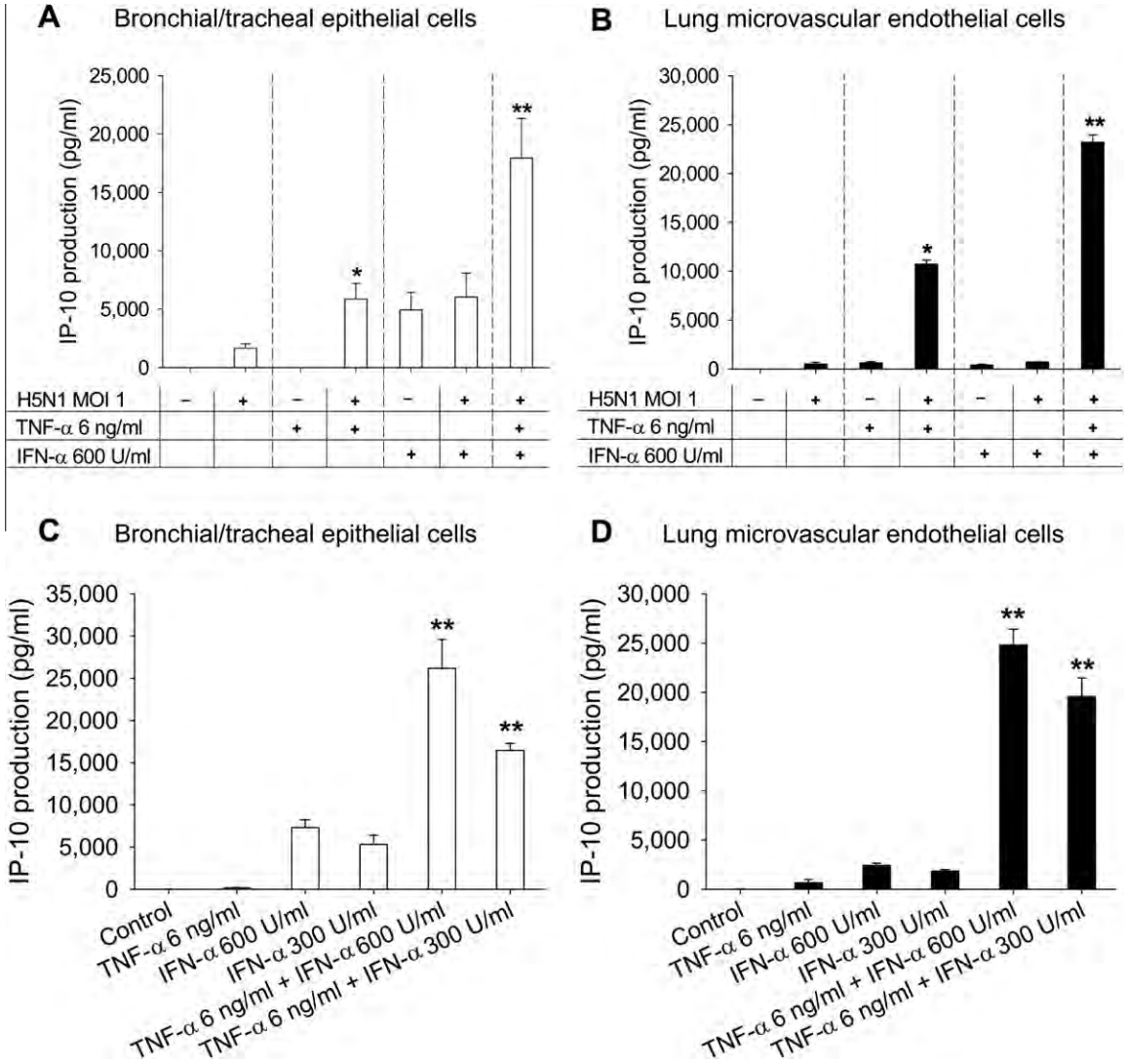

Fig. 3. IP-10 induction with treatment by H5N1, TNF-α, IFN-α, or different combinations in bronchial/tracheal epithelial cells (A) and lung microvascular endothelial cells (B). (C and D) show IP-10 production with treatment of TNF-α and IFN-α in the absence of viral infection. Data are shown as mean values ± SEM of four independent experiments using cells derived from two individual donors. $^{*}P < 0.05$ compared with the sum of the individual effects. $^{**}P < 0.01$ compared with the sum of the individual effects.

3.4. Methylprednisolone was only partially effective in suppression of IP-10 response

In addition to neuraminidase inhibitors, treatment of H5N1 infection often includes corticosteroids to suppress hypercytokinemia. Non-toxic concentrations of glucocorticoid methylprednisolone (4–500 μg/ml) markedly inhibited LPS-induced TNF-α production by PBMC (Fig. 4A). However, in pulmonary cell system, even a high dose of methylprednisolone (100 μg/ml) only partially reduced the synergistic production of IP-10 by TNF-α and H5N1 infection (35% in epithelial and 38% in endothelial cells) (Fig. 4B). Similarly, methylprednisolone only minimally inhibited the IP-10 response induced by the combination of IFN-α and TNF-α (Supplementary Fig. 1).

4. Discussion

We observed increased expression of innate antiviral immune responses in the lung of a fatal H5N1 case, as measured by the expression of IFN-α and MxA protein. Recent observations in mouse model indicate that Mx protein played a critical role in protection against the pandemic 1918 H1N1 and H5N1 viruses [9]. The observed innate antiviral immunity in the H5N1-infected lung in this study seems to be sustained, since IFN-α and MxA could still be detected at day 17 after the onset of illness. The source of this IFN-α is not clear. Preliminary data from our group suggest that lung macrophages are not major IFN-α producer cells, since H5N1-induced IFN-α production was detected in macrophage-depleted lung immune cell cultures (data not shown). Viral infection of the lung often results in early recruitment of plasmacytoid dendritic cells (PDCs) [10]. We hypothesize that PDCs recruited to the H5N1-infected lung are responsible for the observed IFN-α response. This hypothesis is based on our previous findings which showed that PDCs produced very high levels of IFN-α in response to H5N1 viruses [2]. Production of H5N1-induced TNF-α was consistently detected in macrophages [11,12], suggesting that they are the major source of TNF-α. Virus-sensing receptors, including RIG-I, TLR3, TLR7 and NLRP3 inflammasome are important in immune responses against influenza infection [8,13]. Recognition of influenza virus by TLR7 is essential for IFN-α response by PDCs [14]. However, influenza virus-sensing receptor that is responsible for TNF-α production from macrophages remains unclear.

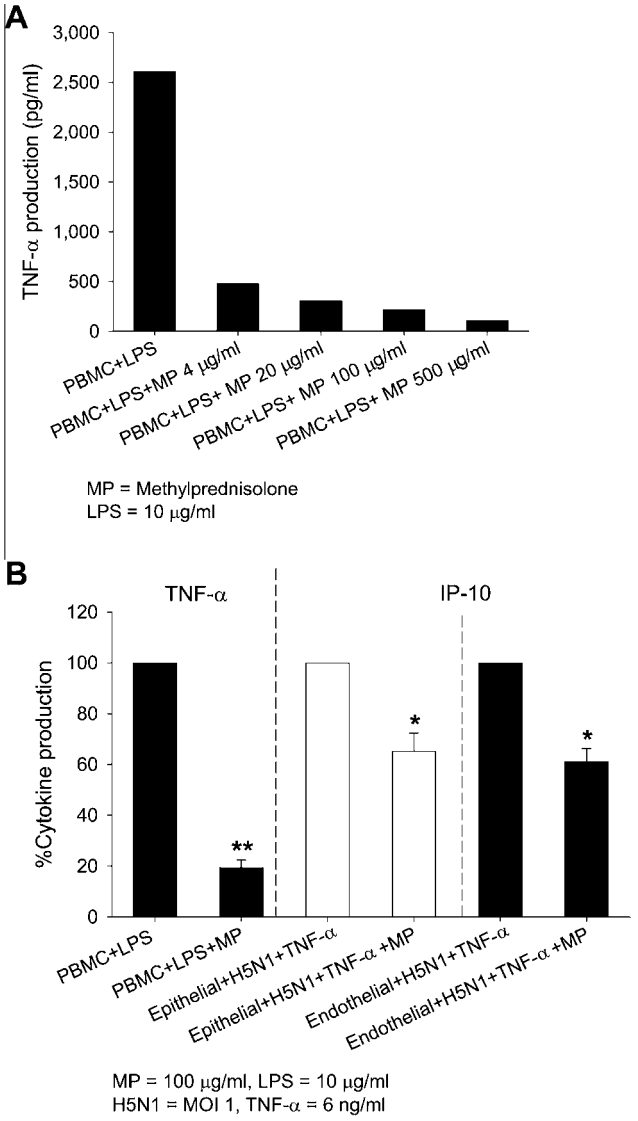

Fig. 4. TNF-α expression in LPS-treated PBMC in the presence of different concentrations of methylprednisolone (MP) (A). Cytokine expression in stimulated PBMC (TNF-α), stimulated bronchial/tracheal epithelial cells (IP-10) and stimulated lung microvascular endothelial cells (IP-10) with and without MP treatment (B). Data in (A) are representative results from one of two experiments. Cytokine expression in treated cells (B) is shown as mean percentage of TNF-α and IP-10 expression ± SEM of four independent experiments using cells derived from two individual donors. $^{*}P < 0.05$ compared with the same cell type without MP treatment. $^{**}P < 0.01$ compared with the same cell type without MP treatment.

It is difficult to obtain autopsy samples from H5N1 patients. We measured expression of IFN-α and MxA in lung tissue from only one case of fatal H5N1 disease. Further research is required to confirm our findings. Nonetheless, our results agree with recent studies in ferret and non-human primate models, which demonstrated that H5N1 infection caused early increase, and sustained type I IFN responses in the infected lung [15,16].

Our observation of IP-10 expression in an H5N1-infected human lung, but not in a control lung, is consistent with a recent study which demonstrated the presence of IP-10 in H5N1-infected human lung [12]. In our study, H5N1 virus was able to infect primary human lung microvascular endothelial cells. The data support recent observations which demonstrated that H5N1 virus could infect human lung endothelial both *in vivo* and *in vitro* [17,18].

In the present study, we showed that H5N1 infection up-regulated RIG-I expression, and modestly up-regulated IP-10 (but not IL-8 and IL-6) in bronchial/tracheal epithelial cells and lung microvascular endothelial cells. Depletion of RIG-I expression led to inhibition of IP-10 response. Because this inhibition was not complete, other pathways are likely to be involved in addition to RIG-I.

The mechanisms underlying the enhanced IP-10 response in H5N1-infected lung are not clear. Treatment with TNF-α greatly enhanced IP-10 production in H5N1-infected bronchial/tracheal epithelial cells and lung microvascular endothelial cells. In contrast, treatment with IFN-α failed to increase IP-10 production in both infected cell types. It has been previously reported that TNF-α or IFN-α primed lung epithelial cell line A549 enhanced H3N2 virus-induced IP-10 expression [19]. H5N1 infection and replication are required to produce enhanced IP-10 response since heat-killed virus showed no effect (data not shown). The quantity of IFN-α (600 U/ml) used in our study was higher than the previous report in epithelial cell line A549 [19] and could inhibit H5N1 replication in human pulmonary cells. This may be the reason why we did not detect an increased IP-10 response when IFN-α was added to H5N1-infected cells.

Combined treatment of cells with IFN-α and TNF-α without viral infection exerted a synergistic effect on IP-10 production, suggesting that uninfected bystander cells could also contribute to hyperchemokinemia in the lung. Our results suggest a complex interplay of H5N1 infection in the lung and locally released TNF-α and IFN-α in the excessive production of IP-10. At least three previous observations have indicated a possible link between IP-10 hyperresponse and severity of H5N1 disease; (1) strong and sustained IP-10 expression in the lung was detected during lethal H5N1 infection in ferrets, compared to H3N2 infection [16]; (2) H5N1-infected non-human primates showed sustained increase in IP-10 response in the infected lung [15]; (3) human data consistently show high blood levels of IP-10 and high plasma levels of IP-10 are strongly associated with fatal outcome in human H5N1 infection [3–5].

IP-10 is well known to chemoattract activated T cells and NK cells by signaling via the chemonkine receptor CXCR3 [20]. Other immune cells, including PDCs, mast cells, infiltrated lung macrophages, and infiltrated lung neutrophils also express CXCR3 [21–24], suggesting that IP-10 may involve in trafficking of these cells to the inflamed lung. The drug AMG487, an antagonist of CXCR3 was recently reported to reduce lung inflammation and increase survival time in H5N1-infected ferrets [16]. Better understanding of the role of IP-10/CXCR3 signaling in pathogenesis of H5N1 disease could well point the way to new and effective therapies.

Glucocorticoids are used in many inflammatory diseases. Our results indicate that methylprednisolone inefficiently blocked TNF-α-mediated IP-10 production by H5N1-infected lung cells. Our findings that methylprednisolone weakly inhibited IP-10 response could partially explain its ineffectiveness in the treatment of H5N1 infection [25]. However, this data should not be over interpreted and more investigations are required.

We hypothesize that initial H5N1 infection in the lung rapidly activates antiviral innate immune responses (IFN-α, MxA, and TNF-α), and IP-10 production. However, the broad tissue tropism of H5N1, combined with its high replication rate, may overwhelm this response. Subsequently, uncontrolled viral infection coupled with locally secreted TNF-α, and IFN-α trigger a massive IP-10 response in infected lungs, promoting inflammatory cells infiltration, resulting in extensive tissue damage. Also, the limited effectiveness of methylpredinisone to inhibit *in vitro* IP-10 response highlights the need for a new and effective anti-inflammatory agent that could be used in conjunction with antiviral drugs to treat H5N1 infection.

Acknowledgments

This work was supported by Grant Y1-AI-5026-01 from the National Institutes of Health, National Institute of Allergy and Infectious Diseases. The views of the authors do not purport to reflect official policy of the U.S. Department of the Army or the Department of Defense.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.07.017.

References

[1] M.C. Chan, C.Y. Cheung, W.H. Chui, S.W. Tsao, J.M. Nicholls, Y.O. Chan, R.W. Chan, H.T. Long, L.L. Poon, Y. Guan, J.S. Peiris, Proinflammatory cytokine responses induced by influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells, Respir. Res. 6 (2005) 135.

[2] A. Thitithanyanont, A. Engering, P. Ekchariyawat, S. Wiboon-ut, A. Limsalakpetch, K. Yongvanitchit, U. Kum-Arb, W. Kanchongkittiphon, P. Utaisincharoen, S. Sirisinha, P. Puthavathana, M.M. Fukuda, S. Pichyangkul, High susceptibility of human dendritic cells to avian influenza H5N1 virus infection and protection by IFN-alpha and TLR ligands, J. Immunol. 179 (2007) 5220–5227.

[3] M.D. de Jong, C.P. Simmons, T.T. Thanh, V.M. Hien, G.J. Smith, T.N. Chau, D.M. Hoang, N.V. Chau, T.H. Khanh, V.C. Dong, P.T. Qui, B.V. Cam, Q. Ha do, Y. Guan, J.S. Peiris, N.T. Chinh, T.T. Hien, J. Farrar, Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia, Nat. Med. 12 (2006) 1203–1207.

[4] J.S. Peiris, W.C. Yu, C.W. Leung, C.Y. Cheung, W.F. Ng, J.M. Nicholls, T.K. Ng, K.H. Chan, S.T. Lai, W.L. Lim, K.Y. Yuen, Y. Guan, Re-emergence of fatal human influenza A subtype H5N1 disease, Lancet 363 (2004) 617–619.

[5] K.F. To, P.K. Chan, K.F. Chan, W.K. Lee, W.Y. Lam, K.F. Wong, N.L. Tang, D.N. Tsang, R.Y. Sung, T.A. Buckley, J.S. Tam, A.F. Cheng, Pathology of fatal human infection associated with avian influenza A H5N1 virus, J. Med. Virol. 63 (2001) 242–246.

[6] M. Uiprasertkul, P. Puthavathana, K. Sangsiriwut, P. Pooruk, K. Srisook, M. Peiris, J.M. Nicholls, K. Chokephaibulkit, N. Vanprapar, P. Auewarakul, Influenza A H5N1 replication sites in humans, Emerg. Infect. Dis. 11 (2005) 1036–1041.

[7] K. Nagata, M. Mibayashi, The Mx protein that confers the resistance to influenza virus, Nippon Rinsho 55 (1997) 2654–2659.

[8] R. Le Goffic, J. Pothlichet, D. Vitour, T. Fujita, E. Meurs, M. Chignard, M. Si-Tahar, Cutting edge: influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells, J. Immunol. 178 (2007) 3368–3372.

[9] T.M. Tumpey, K.J. Szretter, N. Van Hoeven, J.M. Katz, G. Kochs, O. Haller, A. Garcia-Sastre, P. Staeheli, The Mx1 gene protects mice against the pandemic 1918 and highly lethal human H5N1 influenza viruses, J. Virol. 81 (2007) 10818–10821.

[10] M.H. Grayson, M.S. Ramos, M.M. Rohlfing, R. Kitchens, H.D. Wang, A. Gould, E. Agapov, M.J. Holtzman, Controls for lung dendritic cell maturation and migration during respiratory viral infection, J. Immunol. 179 (2007) 1438–1448.

[11] C.Y. Cheung, L.L. Poon, A.S. Lau, W. Luk, Y.L. Lau, K.F. Shortridge, S. Gordon, Y. Guan, J.S. Peiris, Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease?, Lancet 360 (2002) 1831–1837

[12] R. Deng, M. Lu, C. Korteweg, Z. Gao, M.A. McNutt, J. Ye, T. Zhang, J. Gu, Distinctly different expression of cytokines and chemokines in the lungs of two H5N1 avian influenza patients, J. Pathol. 216 (2008) 328–336.

[13] T. Ichinohe, I.K. Pang, A. Iwasaki, Influenza virus activates inflammasomes via its intracellular M2 ion channel, Nat. Immunol. 11 (2010) 404–410.

[14] J.M. Lund, L. Alexopoulou, A. Sato, M. Karow, N.C. Adams, N.W. Gale, A. Iwasaki, R.A. Flavell, Recognition of single-stranded RNA viruses by Toll-like receptor 7, Proc. Natl. Acad. Sci. USA 101 (2004) 5598–5603.

[15] C.R. Baskin, H. Bielefeldt-Ohmann, T.M. Tumpey, P.J. Sabourin, J.P. Long, A. Garcia-Sastre, A.E. Tolnay, R. Albrecht, J.A. Pyles, P.H. Olson, L.D. Aicher, E.R. Rosenzweig, K. Murali-Krishna, E.A. Clark, M.S. Kotur, J.L. Fornek, S. Proll, R.E. Palermo, C.L. Sabourin, M.G. Katze, Early and sustained innate immune response defines pathology and death in nonhuman primates infected by highly pathogenic influenza virus, Proc. Natl. Acad. Sci. USA 106 (2009) 3455–3460.

[16] C.M. Cameron, M.J. Cameron, J.F. Bermejo-Martin, L. Ran, L. Xu, P.V. Turner, R. Ran, A. Danesh, Y. Fang, P.K. Chan, N. Mytle, T.J. Sullivan, T.L. Collins, M.G. Johnson, J.C. Medina, T. Rowe, D.J. Kelvin, Gene expression analysis of host innate immune responses during Lethal H5N1 infection in ferrets, J. Virol. 82 (2008) 11308–11317.

[17] M.C. Chan, R.W. Chan, W.C. Yu, C.C. Ho, W.H. Chui, C.K. Lo, K.M. Yuen, Y.I. Guan, J.M. Nicholls, J.S. Peiris, Influenza H5N1 virus infection of polarized human alveolar epithelial cells and lung microvascular endothelial cells, Respir. Res. 10 (2009) 102.

[18] N.T. Liem, N. Nakajima, P. Phat le, Y. Sato, H.N. Thach, P.V. Hung, L.T. San, H. Katano, T. Kumasaka, T. Oka, S. Kawachi, T. Matsushita, T. Sata, K. Kudo, K. Suzuki, H5N1-infected cells in lung with diffuse alveolar damage in exudative phase from a fatal case in Vietnam, Jpn. J. Infect. Dis. 61 (2008) 157–160.

[19] V. Veckman, P. Osterlund, R. Fagerlund, K. Melen, S. Matikainen, I. Julkunen, TNF-alpha and IFN-alpha enhance influenza-A-virus-induced chemokine gene expression in human A549 lung epithelial cells, Virology 345 (2006) 96–104.

[20] V. Booth, D.W. Keizer, M.B. Kamphuis, I. Clark-Lewis, B.D. Sykes, The CXCR3 binding chemokine IP-10/CXCL10: structure and receptor interactions, Biochemistry 41 (2002) 10418–10425.

[21] C.E. Brightling, D. Kaur, P. Berger, A.J. Morgan, A.J. Wardlaw, P. Bradding, Differential expression of CCR3 and CXCR3 by human lung and bone marrow-derived mast cells: implications for tissue mast cell migration, J. Leukoc. Biol. 77 (2005) 759–766.

[22] S. Grumelli, D.B. Corry, L.Z. Song, L. Song, L. Green, J. Huh, J. Hacken, R. Espada, R. Bag, D.E. Lewis, F. Kheradmand, An immune basis for lung parenchymal destruction in chronic obstructive pulmonary disease and emphysema, PLoS Med. 1 (2004) e8.

[23] D. Hartl, S. Krauss-Etschmann, B. Koller, P.L. Hordijk, T.W. Kuijpers, F. Hoffmann, A. Hector, E. Eber, V. Marcos, I. Bittmann, O. Eickelberg, M. Griese, D. Roos, Infiltrated neutrophils acquire novel chemokine receptor expression and chemokine responsiveness in chronic inflammatory lung diseases, J. Immunol. 181 (2008) 8053–8067.

[24] G. Penna, S. Sozzani, L. Adorini, Cutting edge: selective usage of chemokine receptors by plasmacytoid dendritic cells, J. Immunol. 167 (2001) 1862–1866.

[25] I.N. Kandun, H. Wibisono, E.R. Sedyaningsih, Yusharmen, W. Hadisoedarsuno, W. Purba, H. Santoso, C. Septiawati, E. Tresnaningsih, B. Heriyanto, D. Yuwono, S. Harun, S. Soeroso, S. Giriputra, P.J. Blair, A. Jeremijenko, H. Kosasih, S.D. Putnam, G. Samaan, M. Silitonga, K.H. Chan, L.L. Poon, W. Lim, A. Klimov, S. Lindstrom, Y. Guan, R. Donis, J. Katz, N. Cox, M. Peiris, T.M. Uyeki, Three Indonesian clusters of H5N1 virus infection in 2005, N. Engl. J. Med. 355 (2006) 2186–2194.

Reprint form The American Journal of Tropical Medicine and Hygiene

Am. J. Trop. Med. Hyg., 00(0), 2013, pp. 000–000
doi:10.4269/ajtmh.13-0151

Short Report: Pre-Existing Cross-Reactive Antibodies to Avian Influenza H5N1 and 2009 Pandemic H1N1 in US Military Personnel

Sathit Pichyangkul,* Somporn Krasaesub, Anan Jongkaewwattana, Arunee Thitithanyanont, Suwimon Wiboon-ut, Kosol Yongvanitchit, Amporn Limsalakpetch, Utaiwan Kum-Arb, Duangrat Mongkolsirichaikul, Nuanpan Khemnu, Rangsini Mahanonda, Jean-Michel Garcia, Carl J. Mason, Douglas S. Walsh, and David L. Saunders

Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand; National Center for Genetic Engineering and Biotechnology, Pathum Thani, Thailand; Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand; Department of Dentistry, Chulalongkorn University, Bangkok, Thailand; Hong Kong University—Pasteur Research Center, Hong Kong

Abstract. We studied cross-reactive antibodies against avian influenza H5N1 and 2009 pandemic (p) H1N1 in 200 serum samples from US military personnel collected before the H1N1 pandemic. Assays used to measure antibodies against viral proteins involved in protection included a hemagglutination inhibition (HI) assay and a neuraminidase inhibition (NI) assay. Viral neutralization by antibodies against avian influenza H5N1 and 2009 pH1N1 was assessed by influenza (H5) pseudotyped lentiviral particle-based and H1N1 microneutralization assays. Some US military personnel had cross-neutralizing antibodies against H5N1 (14%) and 2009 pH1N1 (16.5%). The odds of having cross-neutralizing antibodies against 2009 pH1N1 were 4.4 times higher in subjects receiving more than five inactivated whole influenza virus vaccinations than those subjects with no record of vaccination. Although unclear if the result of prior vaccination or disease exposure, these pre-existing antibodies may prevent or reduce disease severity.

Outbreaks of 1997 avian influenza H5N1 and 2009 pandemic (p) H1N1 in humans have provided an opportunity to gain insight into cross-reactive immunity. The US military periodically collects and stores serum samples from service members linked to medical records.[1] We measured cross-reactive antibodies in stored serum to avian influenza H5N1 and 2009 pH1N1 from US military personnel and identified factors associated with presence of neutralizing antibodies.

Two hundred archived serum samples were obtained from the US Department of Defense Serum Repository. They were representative of a wide cross-section of active military personnel at the times of collection, whereas specific geographic information was not available on the individual selected; the cohort represents the general US military population, which is deployed throughout the United States and globally. Fifty samples each were selected from four birth cohorts: (1) < 1949, (2) 1960–1965, (3) 1966–1971, and (4) 1972–1977. Within each cohort, 25 samples were collected in the year 2000 (before the introduction of intranasal live attenuated influenza vaccine [LAIV]), and 25 samples were collected in 2008 (where 51% of donors had received LAIV). It has been suggested that LAIV elicits cross-reactive immunity.[2,3] The samples were all collected before the outbreak of 2009 pH1N1, and there have not been any reported outbreaks of H5N1 in US military personnel.

Assays used to measure antibodies included a hemagglutination inhibition (HI) assay and a neuraminidase inhibition (NI) assay.[4] Viral neutralization by antibodies against H5N1 and 2009 pH1N1 was assessed by influenza (H5) pseudotyped lentiviral particle-based (H5pp)[5] and microneutralization assays, respectively. Electronic medical and vaccination records from the Defense Medical Surveillance System (DMSS), which captured records before the serum sample date, were linked to samples and compared with the *in vitro* results.[1]

The odds ratios (ORs) and 95% confidence intervals (95% CIs) of univariate and multivariate binary logistic regression analyses were used to determine the association between donor characteristics and positive antibody responses. A multiple logistic regression model was constructed, and it included independent variables with a P value of < 0.05 in univariate logistic regression. A P value of < 0.05 was considered to indicate statistical significance. SPSS 12.0 for Windows (SPSS Inc.) was used to perform all statistical analysis. AU1

Cross-reactivity is summarized in Table 1. Although HI assay titers to H5N1 were uniformly low (0.5%), neutralizing antibodies were considerably higher: 14% for the more sensitive H5pp assay[5] and 22.5% for the NI assay. H5pp and NI antibody titers to H5N1 were evenly distributed among birth cohorts and did not differ substantially based on history of vaccination or prior respiratory infections. Of those individuals with neutralizing antibodies to H5N1 (N = 28), 32.1% also had neutralizing antibodies to pH1N1, whereas 19.3% of those individuals with any H5N1-specific antibody response also had neutralizing antibodies to pH1N1 (Table 1). T1

As with H5N1, samples with positive HI titers were low for 2009 pH1N1 at 5.5%, whereas neutralizing antibody titers were higher, with 16.5% positive in the microneutralization assay but only 9% positive in the NI assay. Positive neutralization titers were less evenly distributed among birth cohorts, with only 4% positive in the 1972–1977 birth cohort, whereas 30% were positive in the 1960–1965 cohort. Like H5N1, positive antibody titers to 2009 pH1N1 did not differ substantially based on history of vaccination or prior respiratory infections. Of those individuals with neutralizing antibodies to pH1N1 (N = 33), 27.3% also had neutralizing antibodies to H5N1, whereas 28.9% of those individuals with any pH1N1-specific antibody response also had neutralizing antibodies to H5N1.

Univariate associations between the prevalence of cross-reactive antibodies to H5N1 and 2009 pH1N1 and independent variables, including year of birth, serum collection year, sex, and seasonal influenza vaccination history, are shown in Table 2. The odds of having cross-neutralizing antibodies against 2009 pH1N1 were threefold higher in those donors who received inactivated whole influenza virus vaccine more than five times than those donors with no record of vaccination (95% CI = 1.1–8.9). In relation to subjects born between T2

*Address correspondence to Sathit Pichyangkul, USAMC—Armed Forces Research Institute of Medical Sciences, Department of Immunology and Medicine, 315/6 Rajvithi Road, Phayathai, Bangkok 10400, Thailand. E-mail: SathitP@afrims.org

TABLE 1

Serum cross-reactivity to avian influenza H5N1and 2009 pH1N1 indicating the percentage of subjects considered to have positive titers and the geometric mean titers for each assay

Characteristics (*n*)	H5N1			2009 pH1N1§		
	HI assay* % positive (GM titer)	H5pp† % positive (GM titer)	NI assay‡ % positive (GM titer)	HI assay % positive (GM titer)	Neutralization % positive (GM titer)	NI assay % positive (GM titer)
Total						
200	0.5 (5.1)	14.0 (21.4)	22.5 (121.6)	5.5 (7.1)	16.5 (20.4)	9.0 (92.8)
Birth cohort						
1936–1949 (50)	2.0 (5.3)	18.0 (22.0)	24.0 (126.0)	6.0 (7.3)	16.0 (19.5)	12.0 (97.6)
1960–1965 (50)	0.0 (5.0)	16.0 (20.3)	26.0 (129.6)	6.0 (7.7)	30.0 (27.5)	6.0 (90.3)
1966–1971 (50)	0.0 (5.0)	12.0 (23.3)	20.0 (117.9)	10.0 (8.0)	16.0 (23.6)	10.0 (92.2)
1972–1977 (50)	0.0 (5.3)	10.0 (20.0)	20.0 (113.7)	0.0 (5.7)	4.0 (13.6)	8.0 (91.5)
Serum collection year						
Y2000 (100)	0.0 (5.1)	15.0 (21.7)	21.0 (120.3)	7.0 (7.3)	16.0 (20.6)	11.0 (94.5)
Y2008 (100)	1.0 (5.2)	13.0 (21.1)	24.0 (123.0)	4.0 (7.0)	17.0 (20.1)	7.0 (91.2)
Sex						
Female (32)	3.1 (5.7)	21.9 (26.3)	12.5 (102.4)	3.1 (6.9)	12.5 (19.2)	6.3 (96.7)
Male (168)	0.0 (5.0)	12.5 (20.5)	24.4 (125.7)	6.0 (7.2)	17.3 (20.6)	9.5 (92.1)
Any cross-reactive antibody to						
H5N1 (57)				8.8 (8.9)	19.3 (25.2)	22.8 (119.9)
pH1N1 (45)	2.2 (5.3)	28.9 (31.2)	37.8 (165.2)			
Neutralizing antibodies to						
H5N1 H5pp (28)				10.7 (9.5)	32.1 (33.6)	25.0 (116.9)
2009 pH1N1 neutralization (33)	3.0 (5.4)	27.3 (28.9)	30.3 (140.3)			
Lifetime seasonal vaccinations						
No record (66)	0.0 (5.1)	10.6 (20.2)	27.7 (128.1)	7.6 (7.4)	15.2 (20.6)	12.1 (96.5)
1–5 vaccinations (88)	1.1 (5.2)	15.9 (21.5)	17.0 (109.2)	5.7 (7.1)	17.0 (20.5)	6.8 (89.1)
> 5 vaccinations (46)	0.0 (5.1)	15.2 (22.2)	32.6 (138.8)	2.2 (6.8)	17.4 (19.7)	8.7 (95.0)
Time since last vaccine						
No record (66)	0.0 (5.1)	10.6 (20.2)	22.7 (128.1)	7.6 (7.4)	15.2 (20.6)	12.1 (96.5)
≤ 1 year (96)	0.0 (5.1)	15.6 (21.5)	24.0 (120.7)	4.2 (7.1)	19.8 (21.0)	8.3 (91.2)
> 1 year (38)	2.6 (5.3)	15.8 (22.4)	18.4 (113.4)	5.2 (6.8)	10.5 (18.3)	5.3 (90.6)
Vaccination history lifetime (at least one dose)						
No record of vaccination (66)	0.0 (5.1)	10.6 (20.2)	22.7 (128.1)	7.6 (7.4)	15.2 (20.6)	12.1 (96.5)
Inactivated whole virus (71)	0.0 (5.0)	14.1 (20.4)	22.5 (115.7)	2.8 (6.4)	15.5 (19.6)	5.6 (87.1)
Split type (102)	1.0 (5.0)	15.7 (20.4)	21.6 (115.7)	4.9 (6.4)	19.6 (19.6)	6.9 (87.1)
Influenza vaccine not otherwise specified (16)	0.0 (5.2)	12.5 (27.9)	37.5 (166.4)	0.0 (6.2)	6.3 (16.1)	12.5 (102.3)
Live attenuated intranasal (50)	0.0 (5.1)	10.0 (18.8)	20.0 (112.2)	4.0 (7.0)	18.0 (20.3)	4.0 (85.2)
History of respiratory illness						
No record of illness (119)	0.0 (5.0)	10.1 (18.5)	18.5 (112.6)	4.2 (7.0)	15.1 (20.5)	8.4 (90.7)
Influenza-like illness (4)	0.0 (5.0)	25.0 (20.7)	0.0 (80.0)	0.0 (8.4)	25.0 (28.3)	25.0 (100.2)
Upper respiratory infection (65)	1.5 (5.4)	23.1 (29.3)	27.7 (135.0)	7.7 (7.3)	18.5 (20.7)	9.2 (93.1)
Lower respiratory infection (37)	2.7 (5.6)	18.9 (30.2)	35.1 (157.6)	8.1 (8.1)	21.6 (22.4)	13.5 (108.4)
Respiratory illness past year (28)	0 (5.1)	25.0 (25.1)	32.1 (154.9)	7.1 (8.0)	28.6 (24.4)	3.6 (86.3)

Titers with a value of zero (below the detection limit) were assigned a value of five for calculation of geometric means (GMs).
*H5N1, A/Vietnam/1203/2004; positive titer ≥ 40.
†H5 hemagglutinin (A/Cambodia/408008/05) pseudotyped lentiviral particle; positive titer ≥ 160.
‡Reassortant H1N1 (HA, PB1, PB2, PA, NP, and M from H1N1 [A/PR/8/34]; N1 from H5N1 [A/Vietnam/DT-036/2005]); positive titer ≥ 160.
§2009 H1N1, A/California/04/2009; same positive titer cutoffs as for H5N1.

1972 and 1979, the odds of having cross-reactive neutralizing antibodies against 2009 pH1N1 were significantly higher in subjects born from 1960 to 1965 (OR = 10.3; 95% CI = 2.2–47.9). In a multiple logistic regression model adjusted for serum collection year, birth cohort, and frequency of receiving inactivated whole virus vaccine, birth cohorts, including 1960–1965 (adjusted OR = 11; 95% CI = 2.3–52.9), and a history of more than five inactivated whole influenza virus vaccinations (adjusted OR = 4.4; 95% CI = 1.3–15.6) were statistically significant covariates associated with higher positivity rates for pH1N1neutralizing antibodies. Within these groups, we found no convincing data to suggest that the specific viral compositions in influenza vaccines have an effect on cross-reactive neutralizing antibodies to pH1N1 (data not shown). No statistically significant associations were observed between the prevalence of cross-reactive antibodies to H5N1 and independent variables in univariate or multivariate analyses.

To the best of our knowledge, the present study is the first report of cross-reactive antibodies to both H5N1 and 2009 pH1N1 in a US military population. Cross-reactive antibodies to both influenza viruses were common in this population. Most serum samples (86%) positive in the H5N1 neutralization assay had no detectable HI activity (titer ≥ 10), whereas 94% of samples that neutralized 2009 pH1N1 also had detectable HI activity (titer ≥ 10; data not shown). In addition, cross-reactive antibodies to avian influenza H5N1 were not necessarily accompanied by cross-reactive antibodies to 2009 pH1N1. Taken together, these findings suggest that the observed cross-reactive neutralization against the two influenza viruses was caused by different antibodies in serum samples.

This report is also the first report to associate history of receiving more than five doses of inactivated whole influenza virus vaccine with neutralizing antibodies against 2009 pH1N1. This finding suggests a protective advantage of repeated vaccination with seasonal whole virus vaccine, generating cross-reactive antibodies against previously unencountered strains. It has been suggested that the high immunogenicity of the inactivated whole virus vaccine is partly caused by the adjuvant

TABLE 2
Analysis of characteristics associated with cross-reactive neutralizing antibodies against 2009 pH1N1 and H5N1 AU3

Characteristic (*n*)	2009 pH1N1			H5N1	
	Prevalence	OR (95% CI)	Adjusted OR (95% CI)	Prevalence	OR (95% CI)
Positive neutralizing antibody	33 (16.5%)			28 (14.0%)	
Serum collection year					
2000 (100)	16 (16.0%)	Reference	Reference	15 (15.0%)	Reference
2008 (100)	17 (17.0%)	1.1 (0.5–2.3)	0.7 (0.3–1.8)	13 (13.0%)	0.9 (0.4–1.9)
Birth cohort					
1936–1949 (50)	8 (16.0%)	4.6 (0.9–22.7)	5.3 (1.0–27.0)	9 (18.0%)	2.0 (0.6–6.4)
1960–1965 (50)	15 (30.0%)	**10.3 (2.2–47.9)**	**11.0 (2.3–52.9)**	8 (16.0%)	1.7 (0.5–5.7)
1966–1971 (50)	8 (16.0%)	4.6 (0.9–22.7)	5.1 (1.0–26.2)	6 (12.0%)	1.2 (0.4–4.3)
1972–1977 (50)	2 (4.0%)	Reference	Reference	5 (10.0%)	Reference
Sex					
Female (32)	4 (12.5%)	Reference		7 (21.9%)	Reference
Male (168)	29 (17.3%)	1.5 (0.5–4.5)		21 (12.5%)	0.5 (0.2–1.3)
Positive neutralizing antibody titers					
H5pp (57)	11 (19.3%)	1.3 (0.6–2.9)			
pH1N1 (45)				13 (28.9%)	3.8 (1.6–8.7)
Vaccination record					
Number of seasonal influenza vaccinations					
No record (66)	10 (15.2%)	Reference		7 (10.6%)	Reference
1–5 vaccinations (88)	15 (17.0%)	1.2 (0.5–2.8)		14 (15.9%)	1.6 (0.6–4.2)
> 5 vaccinations (46)	8 (17.4%)	1.2 (0.4–3.3)		7 (15.2%)	1.5 (0.5–4.7)
Time since last vaccination					
No record (66)	10 (15.2%)	Reference		7 (10.61%)	Reference
≤ 1 year (96)	19 (19.8%)	1.4 (0.6–3.2)		15 (15.6%)	1.6 (0.6–4.1)
> 1 year (33)	4 (10.5%)	0.7 (0.2–2.3)		6 (15.8%)	1.6 (0.5–5.1)
Number of inactivated whole virus vaccinations					
No record (129)	22 (17.1%)	Reference	Reference	18 (14.0%)	Reference
1–5 vaccinations (53)	4 (7.5%)	0.4 (0.1–1.2)	0.4 (0.1–1.4)	7 (13.2%)	0.9 (0.4–2.4)
> 5 vaccinations (18)	7 (38.9%)	**3.1 (1.1–8.9)**	**4.4 (1.3–15.6)**	3 (16.7%)	1.2 (0.3–4.7)
Time since last inactivated whole virus vaccination					
No record (129)	22 (17.1%)	Reference		18 (14.0%)	Reference
≤ 1 year (19)	4 (21.1%)	1.3 (0.4–4.3)		3 (15.8%)	1.2 (0.3–4.4)
> 1 year (52)	7 (13.5%)	0.8 (0.3–1.9)		7 (13.5%)	1.0 (0.4–2.5)
Number of split type vaccinations					
No record (98)	13 (13.3%)	Reference		12 (12.2%)	Reference
1–5 vaccinations (94)	19 (20.2%)	1.7 (0.8–3.6)		14 (14.9%)	1.3 (0.6–2.9)
> 5 vaccinations (8)	1 (12.5%)	0.9 (0.1–8.2)		2 (25.0%)	2.4 (0.4–13.2)
Time since last split type vaccination					
No record (98)	13 (13.3%)	Reference		12 (12.2%)	Reference
≤ 1 year (44)	10 (22.7%)	1.9 (0.8–4.8)		10 (22.7%)	2.1 (0.8–5.3)
> 1 year (58)	10 (17.2%)	1.4 (0.6–3.3)		6 (10.3%)	0.8 (0.3–2.3)
Number of intranasal LAIV vaccinations					
No record (150)	24 (16.0%)	Reference		23 (15.3%)	Reference
1–5 vaccinations (50)	9 (18.0%)	1.2 (0.5–2.7)		5 (10%)	0.6 (0.2–1.7)
Time since last intranasal LAIV vaccination					
No record (150)	24 (16.0%)	Reference		23 (15.3%)	Reference
≤ 1 year (34)	7 (20.6%)	1.4 (0.5–3.5)		3 (8.8%)	0.5 (0.2–1.9)
> 1 year (16)	2 (12.5%)	0.8 (0.2–3.5)		2 (12.5%)	0.8 (0.2–3.7)

effect of the viral RNA presented, stimulating innate immunity through the TLR 7-dependent pathway.[6] We hypothesize that the combined effect of adjuvant activity and the heterogenous mix of flu strains that an individual would be exposed to over the course of multiple seasonal vaccinations may enhance the breadth of antibody response and promote the generation of cross-reactive antibodies.

A retrospective case-control study conducted in US military personnel after the outbreak of 2009 pH1N1 showed that both 2008–2009 seasonal influenza vaccine and history of seasonal vaccine in the prior 4 years afforded some protection against pH1N1. Vaccine effectiveness (VE) was high in persons ≥ 40 (55%) or < 25 (50%) years of age but very low in persons 25–39 years of age (< 10%).[7] These findings correlate with the high levels of cross-reactive 2009 pH1N1 antibodies reported here, with 30% in the 1960–1965 cohort (age range = 35–48) but only 4% in the 1972–1977 cohort (age range = 23–36). Our findings are similar to the results found recently in an elderly population in the United States.[8] The exception is in those individuals born before 1950, in whom antibody responses were much higher in this cohort. Both our study and the US study differ from two recent seroprevalence studies in Singapore and China, where cross-reactive antibodies were rare in various age groups.[9,10] High seasonal influenza vaccination rates in US military personnel found here and prior studies[11] may explain the differences observed in these populations, although results from small retrospective seroprevalence studies should be interpreted cautiously. Possible alternative explanations include differences in laboratory assay methods, natural influenza exposure in the sampled populations, and/or use of convenience sampling methods.

Studies in humans suggest that the antibody to influenza neuraminidase is associated with resistance to influenza.[12] A recent serological study in a small number of human serum

samples showed that 24% had cross-reactive antibodies to avian N1,[13] similar to our findings (22.5%). In addition, we observed that 9% of serum samples had cross-reactive antibodies to pH1N1.

Like pH1N1, persons < 40 years old seem to be most affected by H5N1 infection, with infection rarer in older individuals.[14] However, we did not find a difference in cross-reactive antibody prevalence to either neuraminidase or neutralizing antibodies (H5pp) with year of birth or other immunologic markers of exposure, including vaccination history or prior respiratory illness.

A possible limitation of our study is that the DMSS may not have captured all relevant medical encounter and/or vaccination data, particularly for encounters that were not entered into the system electronically or coded accurately. Data in the DMSS are provider-dependent, and the DMSS captures data from various historical time periods, dating back to 1980 for immunization data, 1985 for Department of Defense Serum Repository specimens, 1990 for demographic data, and only 1996 for outpatient data. Interpretation of data presented on history of respiratory illness, which is entirely dependent on voluntary provider reporting and ICD-9 coding, is particularly limited by lack of virologic confirmation.

Cross-reactive immunity to pathogenic influenza strains was found in a subset of US military service members, and it may serve to prevent or reduce the severity of influenza. A better understanding of the mechanisms underlying the development of cross-reactive antibodies will aid in the development of more effective preventive and therapeutic measures.

Received March 22, 2013. Accepted for publication July 21, 2013.

Acknowledgments: The authors thank Dr. Angelia Eick for providing serum samples from the Department of Defense serum repository. We also thank Drs. Malik Peiris, Richard Jarman, and Gray Heppner for their support.

Financial support: This research was supported by National Institutes of Health, National Institute of Allergy and Infectious Diseases Grant Y1-AI-5026-01, the Thailand Research Fund, and Chulalongkorn University.

Disclaimer: The views of the authors do not purport to reflect official policy of the US Department of the Army or the Department of Defense.

Authors' addresses: Sathit Pichyangkul, Somporn Krasaesub, Kosol Yongvanitchit, Amporn Limsalakpetch, Utaiwan Kum-Arb, Duangrat Mongkolsirichaikul, Nuanpan Khemnu, Carl J. Mason, Douglas S. Walsh, and David L. Saunders, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand, E-mails: SathitP@afrims.org, SompornK@afrims.org, KosolY@afrims.org, AmpornL@afrims.org, UtaiwanS@afrims.org, DuangratM@afrims.org, NuanpanK@afrims.org, Carl.Mason@afrims.org. douglas.s.walsh@us.army.mil, and David.Saunders@afrims.org. Anan Jongkaewwattana, National Center for Genetic Engineering and Biotechnology, Pathum Thani, Thailand, E-mail: anan.jon@biotec.or.th. Arunee Thitithanyanont and Suwimon Wiboon-ut, Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand, E-mails: scaty@mahidol.ac.th and swiboonut@gmail.com. Rangsini Mahanonda, Department of Dentistry, Chulalongkorn University, Bangkok, Thailand, E-mail: rangsini.m@chula.ac.th. Jean-Michel Garcia, Hong Kong University—Pasteur Research Center, Hong Kong, E-mail: jmgarcia@hku.hk.

REFERENCES

1. Rubertone MV, Brundage JF, 2002. The Defense Medical Surveillance System and the Department of Defense serum repository: glimpses of the future of public health surveillance. *Am J Public Health 92:* 1900–1904.
2. Lee MS, Yang CF, 2003. Cross-reactive H1N1 antibody responses to a live attenuated influenza vaccine in children: implication for selection of vaccine strains. *J Infect Dis 188:* 1362–1366.
3. Mendelman PM, Rappaport R, Cho I, Block S, Gruber W, August M, Dawson D, Cordova J, Kemble G, Mahmood K, Palladino G, Lee MS, Razmpour A, Stoddard J, Forrest BD, 2004. Live attenuated influenza vaccine induces cross-reactive antibody responses in children against an a/Fujian/411/2002-like H3N2 antigenic variant strain. *Pediatr Infect Dis J 23:* 1053–1055.
4. Lambre CR, Terzidis H, Greffard A, Webster RG, 1990. Measurement of anti-influenza neuraminidase antibody using a peroxidase-linked lectin and microtitre plates coated with natural substrates. *J Immunol Methods 135:* 49–57.
5. Garcia JM, Lagarde N, Ma ES, de Jong MD, Peiris JS, 2010. Optimization and evaluation of an influenza A (H5) pseudotyped lentiviral particle-based serological assay. *J Clin Virol 47:* 29–33.
6. Koyama S, Aoshi T, Tanimoto T, Kumagai Y, Kobiyama K, Tougan T, Sakurai K, Coban C, Horii T, Akira S, Ishii KJ, 2010. Plasmacytoid dendritic cells delineate immunogenicity of influenza vaccine subtypes. *Sci Transl Med 2:* 25–24.
7. Johns MC, Eick AA, Blazes DL, Lee SE, Perdue CL, Lipnick R, Vest KG, Russell KL, DeFraites RF, Sanchez JL, 2010. Seasonal influenza vaccine and protection against pandemic (H1N1) 2009-associated illness among US military personnel. *PLoS One 5:* e10722.
8. Hancock K, Veguilla V, Lu X, Zhong W, Butler EN, Sun H, Liu F, Dong L, DeVos JR, Gargiullo PM, Brammer TL, Cox NJ, Tumpey TM, Katz JM, 2009. Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus. *N Engl J Med 361:* 1945–1952.
9. Tang JW, Tambyah PA, Wilder-Smith A, Puong KY, Shaw R, Barr IG, Chan KP, 2010. Crosss-reactive antibodies to pandemic (H1N1) 2009 virus, Singapore. *Emerg Infect Dis 16:* 874–876.
10. Chen H, Wang Y, Liu W, Zhang J, Dong B, Fan X, de Jong MD, Farrar J, Riley S, Smith GJ, Guan Y, 2009. Serologic survey of pandemic (H1N1) 2009 virus, Guangxi Province, China. *Emerg Infect Dis 15:* 1849–1850.
11. Grabenstein JD, Pittman PR, Greenwood JT, Engler RJ, 2006. Immunization to protect the USArmed Forces: heritage, current practice, and prospects. *Epidemiol Rev 28:* 3–26.
12. Murphy BR, Kasel JA, Chanock RM, 1972. Association of serum anti-neuraminidase antibody with resistance to influenza in man. *N Engl J Med 286:* 1329–1332.
13. Sandbulte MR, Jimenez GS, Boon AC, Smith LR, Treanor JJ, Webby RJ, 2007. Cross-reactive neuraminidase antibodies afford partial protection against H5N1 in mice and are present in unexposed humans. *PLoS Med 4:* e59.
14. Smallman-Raynor M, Cliff AD, 2007. Avian influenza A (H5N1) age distribution in humans. *Emerg Infect Dis 13:* 510–512.